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THE THEORIES OF BLOOD COAGULATION[1]

By Jules Bordet

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First of all, I beg of you to excuse my imperfect knowledge of the English language and to accept my best thanks for the honor you have conferred upon me by inviting me to deliver the Herter lectures. I shall try today to give a brief *résumé* of the chief theories which have been held concerning the mechanism underlying the coagulation of the blood. This phenomenon deserves our interest not only because of its physiological importance but also as a striking example of the resources of experimental analysis. It can occur *in vitro*, and this is a very favorable condition for the success of investigation. Nevertheless, and although it has been the subject of innumerable researches, up to the present time, its mystery has not been completely disclosed. You will not expect me to attempt a detailed review of the whole subject. I shall give only such broad outlines as will serve to make clear the modern conceptions which seem to afford the best explanation of this complicated process, and which, therefore, especially deserve our attention.

[1] Lecture I of the Herter Series, delivered before The Johns Hopkins University on Tuesday, October 26, 1920.

It is hardly necessary to remind you that coagulation is nothing else than the aggregation, into meshes of fibrin, of particles of fibrinogen, a substance which, as Fredericq showed 43 years ago, preexists as a dispersed colloid in the circulating plasma. When the blood flows from a wound, the first determining factor which, through successive modifications of the plasma, assures the solidification of fibrinogen, is not infrequently the mixture of the blood with very active principles liberated by the bruised tissues, or in other words, the addition to the blood of tissue extract. But such an influence is an additional one, foreign to the blood itself; hence, limiting the problem, I shall consider here, exclusively, the coagulation that the blood is capable of showing, solely by means of its own substances.

A most important, even a decisive factor of this automatic coagulation, is the contact of a foreign solid body (glass, for instance), which acts only physically by its presence, since it does not liberate any soluble substance. The contact, external factor, brings into activity the internal factor, belonging to the blood, and that is the process through which the principle

directly and immediately responsible for the coagulation, the fibrin-ferment or thrombin, is produced. In fact, the thrombin, which is found in large quantities in the clot or in the serum, does not exist (as Schmidt showed many years ago), in the circulating blood. Consequently, several stages are to be distinguished in the total process, the most important one being the period in which the thrombin appears, the fibrinogen itself playing merely a passive rôle. Fibrinogen can be extracted by special methods and obtained in a fairly pure condition, but it must be kept in mind that the essential problem presented to the physiologist is the coagulation, not of pure fibrinogen, but of the blood considered as a whole, that is, of a very complex medium, cellular and plasmatic, having a definite reaction and definite osmotic pressure, containing numerous constituents, and especially colloids, which presumably are apt to influence each other through molecular adhesion. Coagulation could not be studied without taking into consideration every influence apt to interfere in the phenomenon.

Since the blood of mammals, as a rule, clots promptly, it seemed essential to the success of the investigation to determine how the course of the process could be protracted and, moreover, how it could be stopped at the first period of its evolution, so as to make possible the separation of the cells from the still liquid plasma. Several methods have been devised; I shall mention them very briefly.

Concentrated salts, magnesium sulphate for instance, hinder the coagulation. Common salt especially answers the purpose, being a normal constituent of the organism. Blood from the artery to which from three to five per cent of salt has immediately been added yields by centrifugalization a clear plasma which does not clot so long as the high saline concentration is maintained, but which, when diluted with an amount of distilled water sufficient to reestablish the normal saline content, clots rather quickly. Decalcifying salts, the type of which is sodium oxalate, prevent completely the coagulation, calcium salts being necessary to this phenomenon. By centrifugalization a clear plasma is obtained, which tends to clot when a soluble calcium salt, such as calcium chloride, is restored.

Coagulation is also prevented if to the active contact of glass a contact is substituted, which is not, if we may so express it, felt by the blood or the plasma. A liquid does not feel a wall, I mean does not react physically to it, unless it is capable of adhering to it. Freund was the first to show that blood flowing from the artery does not clot, or at least clots very slowly, when received in a vessel the inside of which has been coated with oil or vaseline. Since it forms a solid coating, paraffin is very suitable to such experiments, and frequently permits the separation of the cells and the plasma by centrifugalization. In conjunction with Gengou I observed that by these means a clear plasma can be obtained and be kept fluid for 24 hours, but that clotting soon occurs when the plasma is brought into contact with glass. This experiment shows that the contact of glass can bring about its effect without the presence of any cells, that is, without any vital interference—we have to do with a physico-chemical phenomenon.

The blood of certain animals, namely, birds and fishes, as Delezenne has shown, clots very slowly by itself, coagulation being greatly hastened by the addition even of traces of tissue extract. Without the help of decalcification or of paraffin coating the blood of a bird will remain fluid for a long time and even yield by centrifugalization a permanently liquid plasma, provided that the tube inserted into the vessel has not been allowed to touch the wound, and consequently no trace of tissue extract has become mixed with the blood. As a matter of fact, this precaution ought to be taken regularly, whatever may be the species of animal under experiment, as it is quite a general rule that tissue extract accelerates coagulation, this auxiliary influence being particularly noted for birds, because this blood is not so capable of spontaneous coagulation as is that of mammals.

Thanks to such methods, the separation of the two constituents, cells and plasma, can be attained before any beginning of coagulation and—let us emphatically insist upon this essential fact—before any appearance of the coagulating principle, thrombin. There is no need to remind you that serum yielded by coagulation contains thrombin.

We must now try to go further and subject plasma and cells to a closer analysis. Let us consider first the plasma. Soluble calcium salts are necessary to coagulation. How do they act? Pekelharing and Hammarsten have shown the essential fact that these salts are not necessary to the transformation of fibrinogen into fibrin under the influence of thrombin, but are indispensable to the formation of the latter, that is, to the production of thrombin from the mother-substances already present in the circulating blood. The production of thrombin is prevented by oxalate, but on the other hand decalcification does not prevent the coagulation of the fibrinogen by completed thrombin. Indeed, it has been proved that blood, oxalated immediately after withdrawal from the artery, remains permanently fluid, no thrombin being ever detected in it, whereas if serum yielded by normally clotted blood be oxalated, this oxalated serum, added to oxalated plasma, causes the coagulation of the latter. From these facts it follows that oxalated plasma is a most suitable reagent for the detection of thrombin in a given liquid; indeed, an estimation of the coagulating power of such a liquid may be made by taking into consideration the quantity of oxalated plasma coagulated by a certain amount of this liquid, or the rapidity of the occurring coagulation. But it must be borne in mind that, at least when present in serum, the activity of a given thrombin depends not only upon its quantity but also upon its age. The capacity of fresh serum to coagulate oxalated plasma decreases very quickly, by a spontaneous attenuation of the thrombin, and this fact affords a possibility of detecting whether a given thrombin has been produced quite recently or some time ago. Several experiments, as we shall see, require such a determination.

Contact is also necessary to coagulation. How does it operate? I showed with Gengou, many years ago, that con-

tact is in a way analogous to calcium, or in other words, that contact with a foreign solid body (paraffin of course excepted) is necessary to the appearance of thrombin, but is not requisite for the coagulating influence of the latter. When blood is received into a paraffined vessel, thrombin is not formed; when received into a glass vessel, thrombin is produced in the zone of contact, a fact which explains why coagulation begins along the wall. But when serum yielded by previously clotted plasma is added to blood or plasma kept in a paraffined vessel, the entire mass rapidly solidifies, the paraffin no longer exerting any inhibiting influence. This experiment explains why blood freshly drawn and placed in a glass vessel coagulates in a mass much more rapidly when shaken.

What then is the origin of thrombin? It does not exist as such in the circulating blood, although the latter contains everything requisite for its production. The circulating blood, therefore, contains the mother substance, or mother-substances, of thrombin, which, for convenience, may be called prothrombin, and which in the early stages of coagulation is converted into thrombin. What is prothrombin?

Sixteen years ago Morawitz made an important discovery in this connection. He found that if crushed tissue, muscle for example, is added to serum yielded by normal coagulation, the coagulating power of this serum towards oxalated plasma is considerably increased. And yet, the extract of tissue by itself does not contain any thrombin, since without the help of serum, it is incapable of coagulating oxalated plasma. Hence, we are forced to conclude that the tissue extract contains something which is not thrombin, but which reacts with the serum so as to produce this active principle. Thence follows at once the hypothesis that thrombin is derived from the interaction of two different substances, the one furnished by the tissue cells, the other by the serum. Undoubtedly, even before the introduction of the tissue extract, a certain amount of thrombin existed in the serum, but it seems as if this fluid contained also an excess of the mother-substance, the latter being capable of reacting with the tissue extract so as to generate a fresh supply of thrombin.

But the question immediately arises whether such an assumption, deduced from experiments in which tissue extract plays an important rôle, may be without further question applied to the automatic coagulation of pure blood. As a matter of fact, it must be kept in mind that injected into the circulation, the tissue extracts are highly toxic, causing sudden death due to intravascular coagulation. Undoubtedly, they contain some coagulating principle foreign to the blood itself. This substance upon which I shall not dwell today, our present task being merely the study of the coagulation of pure blood, in all probability, is of an albuminoid nature, and is markedly thermolabile. It is specifically related to the tissues, does not exist in the blood, and could not be considered as a real mother-substance of thrombin. But we must immediately add that, besides this peculiar principle, the tissue cells, nevertheless, contain one of the two mother-substances of thrombin. This exists also in the blood cells, is of lipoid nature and may be designated *cytozyme*. The other mother-substance, called by us *serozyme*, is furnished by the blood fluid and is present in the serum. But let us see how these determinations have been arrived at?

The assumption that the blood cells furnish one of the mother-substances of thrombin is in perfect accordance with the results of experiments dealing with the part played by those cells, and chiefly by platelets, in the coagulation. Platelets can be easily separated by centrifugalizing oxalated blood at a moderate speed for a short while; being very light, they remain in suspension, whereas red and white corpuscles are thrown down, the turbid supernatant fluid pipetted off being very rich in platelets. Now if such a platelet-plasma is centrifugalized for a long time and at a very high speed, the platelets finally are sedimented and a clear plasma may be obtained from which the platelets have not been thoroughly eliminated, this being impossible, but in which they are present only in small numbers. Comparing these two plasmas, the one very rich, the other very poor in platelets, Lesourd and Pagniez found that after recalcification the former clots rapidly, the latter slowly. Completing these experiments, with Delange, by comparing the coagulating influence, on oxalated plasma, of the two serums yielded respectively by the coagulation of plasma rich in platelets and of plasma poor in platelets, we observed that the former serum contains a much larger quantity of thrombin than the latter. Consequently, the platelets actively participate in the production of the coagulating principle. This fact can be proved more distinctly by the following experiment: A sediment constituted exclusively of these small cells is obtained by vigorously centrifugalizing oxalated plasma, previously freed of its red and white corpuscles, but containing still its platelets. This platelet deposit, thoroughly washed, is emulsified in physiological solution, and one drop of the turbid emulsion thus obtained is added to a certain quantity of a serum which, being derived from the slow coagulation of recalcified exalated plasma that has been freed of its own platelets, is by itself very poor in thrombin. Within 20 or 30 seconds, the mixture becomes capable of coagulating a suitable amount of oxalated plasma almost instantaneously; in other words the reaction of serum with platelets generates an abundant supply of thrombin. It must be pointed out that this experiment closely resembles that of Morawitz except that platelets instead of tissue cells are added to the serum. Tissue cells and platelets both contain one of the generators of thrombin, which may be called *cytozyme*. The second one, the *serozyme*, exists in the serum. It can be easily demonstrated that the reaction between serum and platelets, that is to say, between serozyme and cytozyme, takes place only in the presence of soluble calcium salts, no thrombin appearing if the serum has been decalcified before the introduction of the platelets. Moreover, I shall further insist on the fact that the two substances unite, that they really consummate each other. Indeed, experience shows that when a serum has been treated once with platelets, and has yielded thrombin, this serum is subsequently incapable of reacting with a new amount of platelets, its serozyme having been exhausted by the first reaction. It follows that a serum pro-

duced by the coagulation of a plasma rich in platelets and which of course contains much thrombin, is considerably less rich in serozyme, that is, considerably less capable of reacting with new platelets, than is a serum derived from a plasma deprived of most of its platelets. This is precisely what experience shows. It is, therefore, highly advisable always to employ for serozyme a serum obtained by the coagulation of oxalated plasma which has been carefully freed of its platelets before recalcification.

Serozyme is a thermolabile substance, easily destroyed by heat. No thrombin is produced when platelets are added to serum that has been exposed to a temperature of about 56° C. On the contrary, cytozyme, the active principle of platelets, may be heated to a hundred degrees and even higher without losing its properties; cytozyme is thermostable and, furthermore, can be easily extracted.

A thick emulsion of platelets, treated with a large excess of absolute alcohol, gives an extract from which, by evaporation, a residue is obtained which is soluble in alcohol, ether, toluol and chloroform but insoluble in acetone, thus exhibiting the characteristics of lipoids, and especially of lecithin. This residue acts as a very powerful cytozyme.

As we were able to show eight years ago, traces of this lipoid behave exactly as do platelets, generating thrombin when added to serum, hastening the coagulation of recalcified oxalated plasma or causing the coagulation of spontaneously non-coagulable bird's plasma. The same lipoid, possessing exactly the same properties may be extracted from tissues, from muscle for example. Cytozyme is thus a lipoid.

These facts having been obtained about cytozyme, what is serozyme? Serozyme is certainly furnished by the plasma, not by the cells. Platelets contain cytozyme; they yield thrombin when mixed with serum but they are never able to liberate thrombin when kept in physiological solution or in distilled water, even in the presence of calcium salts. They consequently contain only one of the mother-substances, not both of them. The lability of serozyme towards heat allows us to presume that this substance is of an albuminous nature. Its fragility would be a very serious hindrance to its isolation, but for one of its properties—a really fortunately one. The serozyme shows a strong tendency to adhere to mineral precipitates, barium sulphate or calcium fluoride for example. This is the reason why, as I discovered many years ago with Gengou, those precipitates, added to oxalated plasma, wholly suppress in the latter the property of coagulating by subsequent recalcification: one of the mother-substances, the serozyme, which is absolutely requisite for the production of thrombin and consequently for coagulation, has been entirely removed. I have found more recently, in conjunction with Delange, that tricalcic phosphate is especially powerful as an absorbent. When diluted in physiological solution, this substance gives a rather gelatinous emulsion, a very slight quantity of which, added to blood flowing from the artery, prevents its coagulation. By centrifugalization and pipetting off, a clear plasma is obtained, which remains fluid, even when platelet emulsion, or tissue extract, or lipoidic cytozyme, is added. This is easily understood; both mother-substances, serozyme and cytozyme, are equally necessary to the production of thrombin; it is of no use to add one of them if the other is absent. But such a plasma, which we may for the sake of brevity call "phosphate plasma," clots under the influence of thrombin or, which naturally is the same, when both mother-substances are added. It behaves as an excellent reagent for the detection of thrombin. Since its composition closely resembles that of the original plasma, it may be considered as being fibrinogen dissolved in a normal medium.

But tricalcic prosphate is endowed with a property which is remarkably useful for technical purposes. As is well known, it is capable of being dissolved in physiological solution under the influence of a current of carbonic oxide gas. Consequently, phosphate which has been added to plasma and has absorbed the serozyme, after having been thoroughly washed, can liberate, thanks to its own dissolution, the active principle it had withdrawn. In this way, Delange and myself succeeded in bringing about the isolation of serozyme, which, on the addition of cytozyme extracted from platelets, gave abundant thrombin. Thus in the course of the whole experiment the factors which determine coagulation are in reality subjected to an analysis followed by a synthesis.

As mentioned above, our assumption that serozyme and cytozyme are the generators of thrombin involves the idea that those mother-substances really unite to form a compound, which is thrombin. This ought to be demonstrated also with regard to pure cytozyme, I mean a cytozyme in the condition of a lipoidic extracted matter. If the union really occurs, we may anticipate that, if a given quantity of serozyme be mixed with a sufficient amount of cytozyme, thrombin will be engendered and the serozyme will be exhausted; in other words, it will be no longer capable of yielding fresh thrombin when a new amount of cytozyme is added. Such is indeed the case. Serum yielded by coagulation of recalcified platelet-free oxalated plasma is divided into two parts, lipoidic cytozyme being added to one of them, the other portion being kept as it is. In the tube containing both serum and cytozyme, thrombin appears, the activity of which is very strong at the outset, but decreases, as we know, very rapidly so as to become quite attenuated by the following day. On the next day, lipoidic cytozyme, and, several minutes afterwards, oxalated plasma are added to both tubes. Then the tube which has received cytozyme on the preceding day does not clot at all, or does so only very slowly, whereas the tube, to which cytozyme has just been added for the first time, clots almost instantaneously.

It is clear that such an experiment makes it possible to ascertain whether the same cytozyme, endowed with the same binding properties as the pure lipoid, exists in fresh or heated platelets, or in tissue juice, ground muscle for example. Adequate experiments show that serum which has already reacted with any one of such materials does not generate any more thrombin when subsequently brought into contact with any one of them. For example, serum to which lipoidic cytozyme has been added will not react any more either with the same lipoid, with platelets, or with muscle juice, and conversely.

Without entering into details, I may add that the manner in which the two substances unite closely resembles the mode of union of toxins and antitoxins, in that the process is not governed by the law of strict and constant equivalents, but takes place in varying proportions, thus seeming to result not from true chemical affinities but from contact affinity or molecular adhesion. But another fact, more noteworthy for its bearing upon the underlying mechanism of coagulation is disclosed by the determination of the lapse of time required for the union of both substances.

Serozyme being found in serum may be assumed to exist also in the oxalated plasma from which this serum has been derived. Now if cytozyme and serum are mixed, thrombin appears very quickly; it is only a question of some seconds. But—and this fact is undoubtedly remarkable—if cytozyme be added, not to serum yielded by coagulation of recalcified oxalated plasma, but to an identical oxalated plasma recalcified just before, that is, at a moment when this plasma is still perfectly fluid, the appearance of thrombin is greatly delayed. In other words, serozyme reacts with cytozyme quickly when present in serum, slowly when present in plasma. We thus reach the conclusion that the serozyme does not exist in the same condition in plasma as in serum, that in plasma it is not capable of reacting at once with cytozyme. We may express this fact by saying that plasma contains proserozyme instead of active serozyme, one of the first phenomena of the whole process of coagulation being precisely the conversion of proserozyme, unfit until transformed to unite with cytozyme, into serozyme capable of this reaction.

The notion that in original plasma or in circulating blood, serozyme does not exist as such, that is, does not exhibit affinities toward cytozyme, satisfactorily explains why intravascular injections of the latter substance are, as we found, quite harmless. But the blood of such injected animals shows, when shed within about half an hour after the injection, a strikingly increased tendency to rapid coagulation. This fact as we have pointed out may probably be available for therapeutic purposes in cases of hemorrhage.

Is it now possible to investigate under what influences the proserozyme is converted into serozyme, in other words, under what influences does it acquire the capacity of reacting with cytozyme?

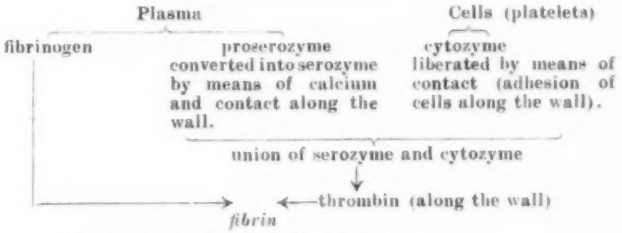
To solve this problem, we have at our disposal a very adequate technic, based on the use of oxalated salt-saturated plasma.

Some minutes ago I mentioned the fact that when oxalated plasma is saturated with common salt, the fibrinogen is entirely precipitated. After strong centrifugalization, pipetting off and elimination of the excess of salt by dialysis in presence of physiological oxalated solution, the supernatant fluid represents exactly normal oxalated plasma, except that, having lost all of its fibrinogen, it cannot coagulate. Being oxalated it does not contain any trace of thrombin, but is still capable of producing plenty of it on the addition of calcium salt and cytozyme. Now if a calcium salt and cytozyme are added thrombin appears indeed, but only after a rather important delay. Half an hour, and sometimes more, must elapse before the mixture becomes capable of bringing about an almost instantaneous coagulation of a fibrinogen solution. Thus the acquisition of the ability to react with cytozyme, that is, the conversion of proserozyme into serozyme, requires a notable length of time. Now, on the other hand, if the aforesaid fluid without fibrinogen is recalcified and cytozyme is added one or two hours later the thrombin appears almost instantaneously. This experiment clearly illustrates the essential assumption that the whole process of the production of thrombin, the first stage included, which is the conversion of proserozyme into serozyme henceforth capable of uniting with cytozyme, takes its course without any participation of fibrinogen.

Furthermore, the conversion of proserozyme, which as we know cannot take place without calcium salts, is—and the fact is noteworthy—strikingly favored by contact with glass. The capacity of reacting with cytozyme appears only after a much longer lapse of time when the recalcified fluid is kept in a vessel coated with paraffin. Consequently, the influence of contact, which is so obvious in coagulation, is not exerted through some interference of fibrinogen, but really acts without any help of the latter, as a factor of thrombin production. It is highly probable that contact, by way of adsorption, frees the liquid of some antagonistic substance, most likely some protective colloid, which prevented the serozyme from reacting with cytozyme, that is, maintained it in the inactive condition of proserozyme. On the other hand, experiments show that the presence of cytozyme likewise facilitates such a liberation of serozyme, owing to its strong affinities towards the latter principle.

To sum up, we are now able to follow the schema which indicates the order of succession of the phenomena.

SCHEMA OF COAGULATION


Plasma
Cells (platelets)
fibrinogen
proserozyme converted into serozyme by means of calcium and contact along the wall.
cytozyme liberated by means of contact (adhesion of cells along the wall).
union of serozyme and cytozyme
thrombin (along the wall)
fibrin
(contact and calcium no longer necessary)

I think that the schema symbolizes quite accurately the most prominent features of the whole process and distinctly shows the sequence of events. But the mechanism underlying coagulation as it occurs under ordinary conditions is still somewhat more complicated, owing to a peculiar property of thrombin. Thrombin results from the union of serozyme and cytozyme, but these two substances combine in variable proportions. The consequence is that a given complex, when rich in serozyme, is able to capture an additional amount of cytozyme, and, when rich in cytozyme, which is ordinarily the case in the coagulation of whole blood, shows a marked affinity towards a new amount of serozyme. As a matter of fact, such an affinity

is so strong that it causes thrombin to attract and to possess itself of serozyme even when this principle is still present in a state of proserozyme. Consequently, finished thrombin acts as though it could bring about a remarkably quick conversion of proserozyme into serozyme, the process preliminary to the genesis of fresh thrombin being thus greatly hastened. The consequence is that, when thrombin is added to oxalated plasma which has been just recalcified, the total amount of thrombin this quantity of plasma is apt to furnish appears much more rapidly than it does when the same plasma is allowed to clot spontaneously without the stimulus of thrombin. In fact, thrombin itself thus accelerates the formation of thrombin. Owing to lack of time, I cannot report here in detail the experiments which have established this idea, but shall now consider briefly some of the views held by certain authors and which are not in agreement with the ideas developed above.

As is well known, my countryman, the physiologist Nolf, has adopted the rather startling theory of Wooldridge according to which, instead of being the immediate determining factor of coagulation, thrombin is generated as a consequence of the coagulation itself. According to Nolf, the transformation of fibrinogen into fibrin is not the effect, but the necessary condition of the appearance of thrombin. Many of the data which I have recorded above energetically plead against such a conception. For example, I would only recall the experiments showing the production of thrombin in fluids altogether devoid of fibrinogen, and thus proving unquestionably that fibrinogen does not play any rôle in the production of the coagulating principle.

One important point has been and still is controverted: I mean the true significance of the lipoid to which we have so often alluded. Schmidt, who had already observed the accelerating influence on coagulation, exerted by alcoholic extracts of tissues, believed that such lipoids rendered easier the production of thrombin, without assuming, as I do, that they really enter into its constitution. One of the most distinguished among the writers who have devoted their skill to the study of coagulation, Professor Howell, especially directed his attention towards the fact that the lipoid extracted, for example, from nervous tissue, is capable of inducing the coagulation of peptone plasma and hirudin plasma which, as is well known, remain fluid because they contain an anticoagulating substance, called antithrombin. In opposition to our assumption, Howell thinks that the lipoid is not a constituent of thrombin, but acts by neutralizing the antithrombin, which hindered the spontaneous conversion of prothrombin into thrombin. The real existence of antithrombin is of course unquestionable and it has been proved beyond doubt that antithrombin may be neutralized by thrombin, the two substances being, in all probability, capable of forming a compound. Now the question arises whether, when lipoid is added to peptone or hirudin plasma, the removal of the antithrombin function is due, as Howell claims, to the direct neutralization of antithrombin by this lipoid, or to a neutralization of antithrombin by thrombin generated under the influence of the same lipoid, the latter reacting with the serozyme or proserozyme also contained in the aforesaid plasma. In other words, according to this second interpretation, the neutralization of antithrombin by the lipoid would be merely apparent or at least indirect, the direct agent of this neutralization being the thrombin which the lipoid has caused to appear. I believe that such a conclusion is forced upon us by some recent and careful experiments by Gratia. Without entering into the somewhat complicated details, they have shown that the lipoid does not at all neutralize the antithrombin when the serozyme or proserozyme has been previously removed, that is, when the production of thrombin has been made impossible. Even when the lipoid is added in large excess, the abolition of the antithrombin function occurs only in proportion to the amount of serozyme present, that is, in proportion merely to the quantity of thrombin that can be generated. Consequently, a direct influence of the lipoid on the antithrombin cannot be admitted.

Furthermore, Howell's view could hardly be brought into harmony with a very essential fact, which has been mentioned above. Were his assumption correct, it should be admitted that serum yielded by the coagulation of recalcified oxalated plasma deprived of its platelets contains a large amount of antithrombin, since the addition of lipoid to such a serum, itself poor in thrombin, produces in this fluid plenty of the latter principle: upon the whole, the serum should in this respect resemble very closely the plasma from which it is derived. But, such being the case, it would be very difficult to understand why the lipoid neutralizes the antithrombin very quickly when added to serum, and very slowly when added to plasma. I think the only possible explanation of such a difference is that in serum but not in plasma, as was said before, the serozyme is capable of reacting very rapidly with cytozyme to generate thrombin. However, the question as to the relation of cytozyme to the antagonistic function is one of the most delicate in the whole study of coagulation and I fully realize that different views may still be upheld. As I told you at the beginning, coagulation has been studied for years and years by many investigators; none of them can presume that the problem is yet solved; every one of them merely indulges in the hope of gathering some complementary data; a little more information.

JOHNS HOPKINS HOSPITAL BULLETIN

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ON CERTAIN VARIATIONS IN THE FORM OF THE HUMAN ELECTROCARDIOGRAM

By EDWARD P. CARTER and FRANCIS R. DIEUAIDE *

(From the Cardiographic Department of The Johns Hopkins Hospital and University)

Einthoven [5] pointed out that the form of the human electrocardiogram varies with respiration and with the position of the body. Waller [7, 8] independently made many observations to the same effect. These variations consist for the most part of changes in the amplitude and sometimes of the direction of the waves of the QRS complex. They have been shown to be related to changes in the electrical axis of the heart in accordance with developments growing out of Einthoven's law that lead II is equal to the sum of leads I and III. The subject from this point of view is summarized in a recent article [3] from this laboratory. The purpose of this communication is to point out more clearly certain peculiarities of these and other variations in the form of the electrocardiogram which have a practical bearing.

The variations which we are to discuss are seen very frequently in lead III and this fact has led to a feeling that the findings in this lead are unimportant, or may even be wholly ignored. Such a policy is not warranted by the explanation of the changes which may occur, and tends to prevent us from gaining the most that can be learned from the interpretation of the electrocardiographic records.

The commonest variation in the electrocardiogram is that the amplitude of R in lead I increases with expiration and decreases with inspiration, whereas R in lead III behaves in the opposite manner (the fact that an S in lead III acts as an algebraically negative R should be kept in mind). As stated above, this conspicuous variation is commonly most marked in lead III and it is often possible, as is well known,[7] to change a positive to a negative wave in this lead by deep respiration or by a change in position of the subject. An example of this reversal of lead III is given in Fig. 1, in which the change is due to a shift of position. In this instance the value of lead III when the patient was on his right side (a) is -5 mm., and when he turned on his left side (b) it is $+5$ mm. (measured at corresponding points of the respiratory cycle).

In examining routine records it was noticed that the same changes are not infrequently present in leads I and II in different cases. So far as is known this has not been commented upon. Fig. 2 is an illustration of reversal of direction in lead I. When the subject was prone (a) the value of lead I (in expiration) is $+5$ mm., and it is -3.5 mm. when the left lateral decubitus is assumed (b). In addition the wave becomes deeply notched. An instance of marked change in lead II appears in Fig. 3. In lead II during one phase the value of R is 13 mm., and during another phase it is 9 mm., while at the same time a marked notching appears. *Variation in amplitude, as might be expected, is much more frequent than reversal of direction; it is also seen not only in lead III but in the other leads in appropriate cases.*

Of the same nature as these peculiarities of the electrocardiogram, at least from our present point of view, is that lead in which there is practically no deflection of the galvanometer string from the iso-electric line during the time corresponding to the QRS interval. This phenomenon is seen not infrequently in one of the three leads, and there are many examples in leads I and II as well as in lead III. Fig. 4 illustrates the phenomenon in each of the three leads (the records are from different individuals). It happened that these records were all taken within a period of five days. It may be remarked that in the presence of this peculiarity the two leads which are not iso-electric, or of very low potential difference, have approximately equal values. It is of importance that the occurrence of these practically zero values, when confined to a single lead, is not in itself of any significance, prognostic or otherwise, as will be readily appreciated from the explanation of the phenomenon given below.

Notching of R or S depends upon the manner in which the electrical axis shifts, as was shown by Williams.[10] Such notching is sometimes transient (see Figs. 2 and 3), and examples of this peculiarity in each lead are fairly numerous. Since the same principle must underlie the phenomenon whether it be transient or persistent, the significance to be attached to notching of a given degree is not decreased by transiency. It also follows that *the emphasis which is to be put on the notching does not depend upon the lead in which it presents itself.* When the principles of the electrical axis are borne in mind, a careful examination of the three leads practically always shows signs in all leads of the factors underlying the notching. Fig. 5 shows progressive change in the notching of leads I and III which is merely a reflection of shifts of the electrical axis. Evidence of the notching is present all through the records and analysis of the shift of the axes shows that the fundamental course of the axis remains the same. The stability of lead II is noteworthy.

When patients are followed by taking electrocardiographic records in the standard method at intervals, similar changes are often observed in one or the other lead.

The mechanism of these changes, as far as it is known, has to do with the position of the electrical axis of the heart during its rotation. When the electrical axis is nearly perpendicular to the plane of a given lead, slight changes in the angle of the axis give rise to relatively wide variations in the value of that lead. For example, a shift of 5° from 80° to 85° halves the value of lead I, while a shift of the same extent from 5° to 10° decreases the amplitude of lead I by only 1.1 per cent (see 3, table 2). The striking changes of reversal of negativity

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illustrated above occur when the electrical axis is very nearly perpendicular to the plane of the lead affected and is carried over to the other side of the perpendicular by the shift which occurs. The appearance and disappearance of notching of R or S is to be explained on the same basis.

In a given case which shows variation in amplitude, reversal in direction of R or S, or transient notching, the peculiarity is marked in one lead and present to a less extent in another, while it is practically absent from the remaining lead. This follows from the relation which exists between the planes of the leads as shown by Einthoven,[4] namely, that of the sides of an equilateral triangle. It is convenient to refer to the lead or leads which show relatively marked changes as *unstable*, while those which do not vary may be said to be *stable*.

The original exposition of this subject by Einthoven[4] sets forth that the leads change their values from positive to negative and vice versa at the following angles of the electrical axis to the horizontal plane: lead I at $+90°$ * and $-90°$; lead II at $+150°$ and $-30°$; and lead III at $+30°$ and $-150°$. When the electrical axis is very close to one of these angles and undergoes but little shifting the corresponding lead is of approximately zero value. Table 1 gives for each

TABLE 1.—SHOWING THE ANGLES BETWEEN WHICH A SHIFT OF 5° CHANGES THE LEAD VALUE 18% OR MORE

Leads	Angles	
I	+ 65° to +115°	—115° to — 65°
II	+125° to +175°	— 55° to — 5°
III	+ 5° to + 55°	—175° to —125°

TABLE 2.—SHOWING THE ANGLES BETWEEN WHICH A SHIFT OF 5° CHANGES THE VALUES OF TWO LEADS AT LEAST 10%

Leads	I+	II+	III+	III—
I+	.....	.....	+50° to +70°	.....
I—	.....	+110° to +130°	.....	—130° to —110°
II—	—70° to —50°	.....	+170° to —170°	.....
III—	.....	—10° to +10°	.....	.....

of the leads the angles between which a shift of the electrical axis to the extent of 5° changes the value of the lead 18 per cent or more. The angles between which a similar rotation of 5° affects the values of two leads at once to the extent of at least 10 per cent are shown in Table 2. These tables are arranged on the basis of the table for the calculation of the "manifest value" recently published from this laboratory (3, Table 2). They are offered with the hope that they may help make useful a knowledge of the peculiarities of the electrocardiogram under discussion.

* The custom of designating the angle "*a*" as positive when below and negative when above the horizontal plane is followed. For methods of determining this angle consult (2) and (3).

Examination of these tables shows why variation of the QRS group is more commonly seen in lead III. It is because normal hearts as a rule have electrical axes which lie between $+30°$ and $+70°$ (although the normal limits are somewhat wider), limits within which lead III varies conspicuously. On the other hand, in cases of ventricular preponderance in which the angle of the electrical axis is negative or greater than $+90°$ it is chiefly lead II which is unstable (see tables). It was essentially for this reason that the suggestion was made[1] to use leads I and III for the determination of the angle of the electrical axis in cases of suspected preponderance.

The electrocardiographic variations described may be divided into two groups, namely, those which occur in a very brief period, often during the inscribing of the record; and those which are first found after the lapse of a relatively long interval.

With regard to the underlying cause of the variations our knowledge is very imperfect. Simple variation in amplitude and change in direction and appearance and disappearance of notching during the taking of the record are no doubt due for the most part to the effect of respiration on the heart, as was long ago pointed out. These changes can also often be brought about in unstable leads by altering the position of the body from the prone to the left or right lateral position. In a few cases such conditions as gastric distention, ascites, hydrothorax and pneumothorax may play a part. Doubtless it is in a sense accidental that the electrical axis in a given instance happens to fall so close to the proper angle as to cause almost no deflection in the corresponding lead.

Marked variations of the nature described, beyond the possibility of explanation on the basis of a change in the relative position of the heart, often occur especially with lapse of a longer interval. These changes are of course, of much greater significance. Their full explanation is wanting as yet. In addition to the occurrence of definite pathological changes in the conduction system, disturbances in the relation between the ventricles as to mass and perhaps as to volume, are thought to play a prominent part. It is quite possible that local metabolic changes may be a factor. It is not to be concluded that the facts presented are supposed to prove a constant relation between the electrical and the anatomical axis of the heart although there is no doubt a definite relation of some nature between them.

It is further of importance to keep in mind the fact that the calculated electrical axis represents essentially the resultant of the constantly shifting axes during the spread of the excitation wave, and does not give us any clue as to the actual degree or direction of the rotation that may have taken place during the inscription of the QRS complex. This can be determined only by exact analysis from given time instants with all three leads accurately in phase.

Emphasis should be laid on the occurrence of these peculiarities in all the leads, and particularly on the fact that they are very often present in lead II in pathological hearts.

The principles applied in this paper to the consideration of the QRS complex in the three leads are also applicable to

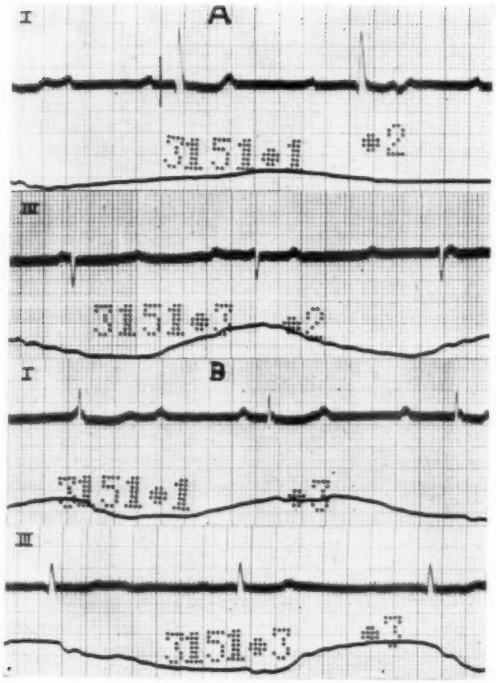

FIG. 1.—Showing alteration in amplitude of lead I and reversal of direction with change of position. (A) Subject lying on the right side; (B) lying on the left side.

FIG. 2.—Showing reversal of direction of lead I and variation in the notching of leads I and II with change of position. Note also the variation in amplitude. (A) subject lying on his back; (B) lying on the left side.

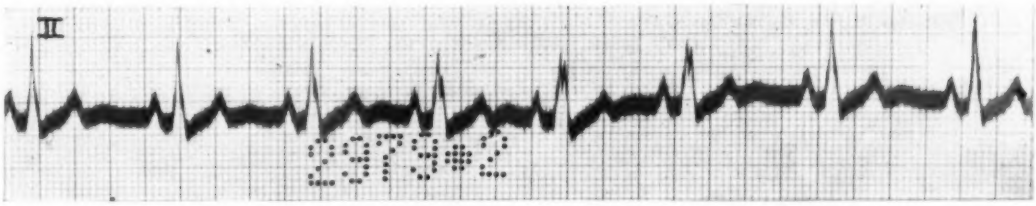

FIG. 3.—Showing variation in amplitude and notching of lead II.

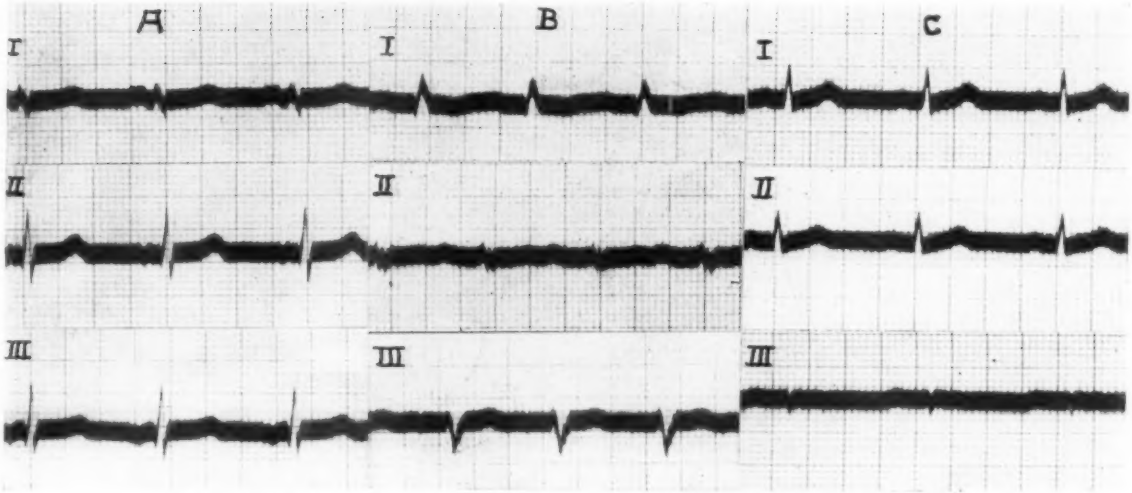

FIG. 4.—Showing leads of approximately zero value: (A) Lead I; (B) lead II, (C) lead III.

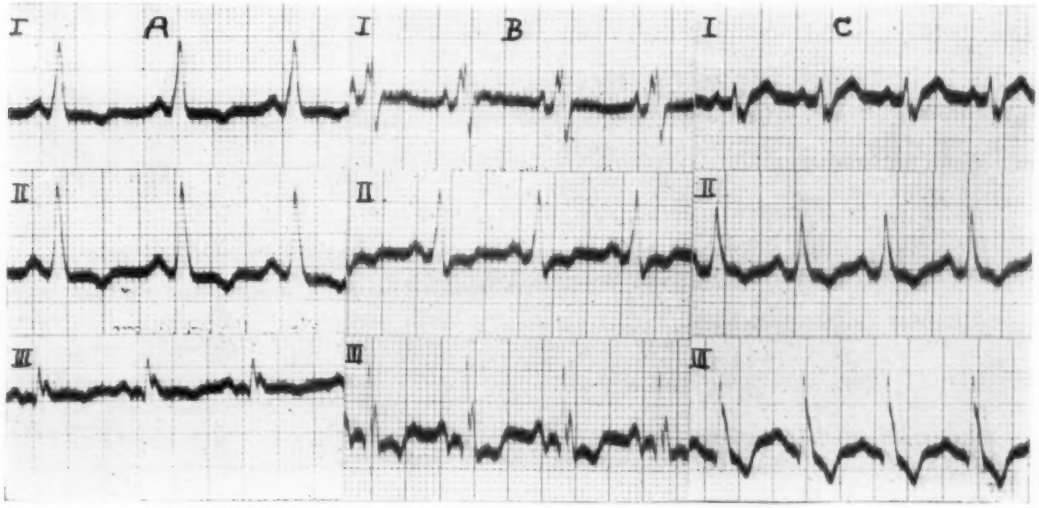

FIG. 5.—Showing progressive change in the notching of leads I and III. Note the stability of lead II. (A) October 28, 1920; (B) February 1, 1921; (C) March 21, 1921.

similar changes undergone by the "P" and "T" waves of the human electrocardiogram. Studies dealing with these latter waves in this connection are under way at the present time in this laboratory.

SUMMARY

1. Variations in amplitude (including extremely low values) and direction of the QRS complex and transient notching of R and S in the human electrocardiogram occur not only in lead III but also not uncommonly in the other leads, especially in records of abnormal hearts.

2. These peculiarities are dependent upon the relation of the electrical axis of the heart to the planes of the leads. Tables are given showing the angles at which conspicuous variation is present in each lead, and the angles between which fairly marked variation occurs in each combination of two leads. The terms "stable" and "unstable" are suggested with reference to the variability of the leads.

3. Variations are due in some instances to shifts in the relative position of the heart; in others to pathological changes in the bundle tract and to alteration in the relation between the ventricles; in a great many others to causes at present unknown.

4. No significance is to be attached to curves of low amplitude in any one lead only.

5. In the light of the evidence set forth above, it is not justifiable to assume that the findings of lead III can be disregarded.

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A STATISTICAL NOTE ON EPIDEMIC ENCEPHALITIS*

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During the last years of the war and since there has occurred the significant beginning and menacingly rapid spread of a disease either previously not existing, or at least not occurring frequently enough to get general recognition, namely, *encephalitis lethargica* or epidemic encephalitis. Because of its extremely rapid development in frequency of occurrence and because of its extremely fatal character, together with the probability that it may become an epidemic disease of an importance similar to that of poliomyelitis it seems desirable, thus early in its history, to make some analysis of its statistical characteristics. To do this has been the object of the preparation of this paper, in which I have been assisted on the arithmetic side by Mr. John Rice Miner.

The aid which the mathematical analysis of statistical data can render the epidemiologist and pathologist, and the degree of certainty which such analysis can add to their conclusions, are not generally recognized. Usually the health officer is content with the mere tabulation of frequency of occurrence of morbidity and mortality. Such figures are too frequently made the basis of conclusions without any recognition of the errors of sampling involved. Before one can be in any degree certain of conclusions from such figures their probable errors must be known.

Material.—Two papers have recently appeared giving raw data regarding the development of epidemic encephalitis. The first of these papers[1] deals with the cases of the disease in the whole United States in the years 1918 and 1919. The second paper[2] deals with the much more restricted field of New York City in 1919 and 1920, but because of the accelerated frequency of occurrence of the disease during the latter year, contains far more statistical material than Smith's paper. It is with the data from this second paper that we shall chiefly deal here.

Case Fatality Rate.—Table 1, taken from (2), gives the incidence and deaths from this disease in New York City in 1919 and 1920. There is a slight correction of an obvious arithmetic error in the total deaths in 1919, from that given in the original.

These data give the following case fatality rates:

$$1919,\ \text{case fatality rate} = \frac{33 \times 100}{128} = 26 \text{ per cent}$$

$$1920,\ \text{case fatality rate} = \frac{211 \times 100}{565} = 37 \text{ per cent}$$

The first of these figures agrees well enough with the 29 per cent given by Smith for the case fatality rate over the whole country in the years 1918 and 1919. The significant thing is that the case fatality rate appears to be increasing. Such a conclusion depends upon the assumption that morbidity from this cause was at least as well reported in 1920 as in

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1919. It may be accepted with reasonable certainty that the *deaths* were as well reported in the one year as the other. The well-known high standards maintained in the work of the Bureau of Vital Statistics of the New York City Department of Health practically ensures this. The morbidity figures are more doubtful, because this disease has only recently been made reportable in the city. But let us carefully examine the alternatives which are presented. If we assume (a) that the deaths have been equally well reported in 1919 and 1920, which is a justifiable assumption, and (b) that the true, but unknown,

TABLE 1.—INCIDENCE AND DEATHS FROM EPIDEMIC ENCEPHALITIS, BY MONTHS, 1919-1920

Month	1919		1920	
	Cases	Deaths	Cases	Deaths
January	5	..	36	12
February	16	..	149	50
March	25	9	116	52
April	10	1	66	16
May	16	5	42	22
June	4	1	20	12
July	2	1	39	9
August	1	..	28	11
September	8	5	22	6
October	23	7	11	3
November	9	3	12	9
December	9	1	24	9
Totals	128	33	565	211

case-fatality rate has remained absolutely constant at the 1919 figure during these two years, then it follows that to get the 37 per cent observed, it will be necessary to suppose that the 565 cases reported in 1920 far *underestimate* the true number which occurred. It must be assumed that actually 812 cases occurred instead of the 565 reported. Now it would seem altogether unlikely, indeed indefinitely improbable, that in the year 1920 there occurred in New York City 247 cases of encephalitis lethargica about which the Department of Health did not know. The *prima facie* reason why this is so improbable is the ever-increasing interest and recognition of the importance of this disease by the Department of Health during the period named.

Suppose we consider the second alternative which is that the figures for 1919 greatly underestimate the true and assumed constant case fatality rate. This means, if we assume the deaths to have been substantially accurately reported for the same reason as before, that there were more *cases* reported in 1919 than actually occurred. To justify this second alternative it must be supposed that actually there occurred in 1919 in New York City only 89 cases of epidemic encephalitis, instead of the 128 reported. This would be a highly improbable assumption. And further we must reckon with the close agreement between Smith's figures of 29 per cent for the case fatality rate for the whole country and the New York 1919 figures of 26 per cent.

Altogether it may be tentatively concluded, with a high degree of probability, that the case fatality rate from the disease is on the increase. But the New York numbers are absolutely small in a statistical sense, and the further question must be asked, as to whether the 11 per cent increase in the case fatality rate between 1919 and 1920 in New York is statistically significant, *i. e.*, greater than might reasonably be supposed to have arisen from chance fluctuation alone.

The problem may be stated in this way: If in a sample of 128 cases of the disease 33 deaths occur, what would be the number of deaths expected in a sample of 565 cases, if nothing but chance were involved in the matter, or in other words if there had been no change in the true case-fatality rate?

We have, by well understood principles (cf. 4 and 5):

Mean deaths expected in second sample of $565 = 148 \pm 16$.

Or, in other words, it is an even chance, if chance alone caused in 1920 fluctuations from the case-fatality rate observed in 1919, that from 132 to 164 deaths would have occurred in 1920, rather than a number smaller than 132 or larger than 164. Actually 211 deaths occurred in 1920. The difference between 211 and 148 is more than 3.9 times the probable error of the latter figure. Whence we may conclude that there was a statistically significant rise in the case fatality rate in 1920 as compared with 1919 in New York City.

Seasonal Distribution.—A question which at once occurs to one in examining Table 1 is this: Is the seasonal distribution (by months) of cases changing as the disease grows in importance, or is the monthly distribution in 1920 different from that of 1919 only by as much as might reasonably be expected from chance sampling?

To determine this question it is necessary only to determine the probability that the two distributions differ significantly from each other. It has been shown by Pearson [5, 6, 7] that such a probability will be given by the expression

$$P = \frac{\int_{\chi}^{\infty} \chi^{q-2} e^{-\frac{1}{2}\chi^2} d\chi}{\int_{0}^{+\infty} \chi^{q-2} e^{-\frac{1}{2}\chi^2} d\chi}.$$

Where

$$\chi^2 = NN'S\left[\frac{(f/N - f'/N')^2}{f+f'}\right]$$

the summation S extending over all pairs of the elemental frequencies f and f'.

From the data of Table 1, we get for the probability that the 1920 and 1919 distribution are two random samples from the same unchanged phenomenal universe

$$\chi^2 = 89.542,$$

whence it is easily determined that

$$P < .000,000,1.$$

Or, in words, it appears that less often than once in 10 million trials would one expect to get true samples of the size here dealt with, as divergent as are the 1919 and 1920 monthly distributions, from the operation of chance alone. Hence we conclude that there was a significant difference in the seasonal incidence of the disease, as indicated by the monthly distribution of cases, in 1919 as compared with 1920 in New York. Examining the details of the χ^2 calculations it is seen that the significant divergences are in the autumn and the spring. There was a significant flare-up in the spring of 1920 lacking in 1919, and a significant autumn outbreak in 1919 lacking in 1920.

Time Lag of Deaths.—On the basis of the 24 months' experience exhibited in Table 1 it is possible to determine the average time that deaths lag behind cases. Put in another way this means the mean duration of *fatal cases* of encephalitis lethargica, between recognized onset and death. This is found to be:

Mean duration of time
between onset and death = 1.046 ± .230 months.

The epidemiological significance of this datum is that it may be expected, in so far as the 1919 and 1920 experience of New York City may be regarded as typical, that the peak of mortality in an epidemic outbreak of this disease will probably occur from 23 to 37 days after the peak of morbidity has been passed. The contrast of this with the time lag in epidemic influenza is sufficiently striking.

Influence of Sex.—Table 2, again taken from (2), gives the sex and age distribution of the cases and deaths in New York City in 1920.

From these data the first question to which attention may be directed is this: Is the difference between the sexes in respect of (a) incidence, or (b) fatality, greater than might reasonably be expected to arise from chance alone, in a city having the sex distribution of its population that New York has?

By elementary principles of probability it is easily found that, among those persons having the disease in New York in 1920, the probability that a given person picked at random would be a male is given by

$$p = \frac{316}{549} = .5756.$$

whence

$$\frac{pq}{n} = .000445.$$

In the whole population of New York (on the basis of the 1910 census figures) the probability that any person picked at random will be a male is

$$p' = .4994 \text{ and}$$
$$\frac{p'q'}{n'} = .000,000,001$$

whence

$$p - p' = .0762 \pm .0142$$

and

$$\text{Diff./P.E. Diff.} = 5.4.$$

Or, it appears that there is a significantly larger proportion of males among those attacked by epidemic encephalitis than there is among the general poulation. A deviation as large as that between p and p' would occur on the basis of chance alone only once in about every 10,000 trials.

TABLE 2.—ENCEPHALITIS CASES AND DEATHS, BY AGE AND SEX, 1920

Ages	Cases			Deaths		
	Male	Female	Total	Male	Female	Total
Under 5 years	23	24	47	10	8	18
5– 9 years.....	26	14	40	10	2	12
10–14 "	32	18	50	6	5	11
15–19 "	23	24	47	5	5	10
20–24 "	37	34	71	13	11	24
25–29 "	29	27	56	11	14	25
30–34 "	35	18	53	8	8	16
35–39 "	30	18	48	14	11	25
40–44 "	19	14	33	7	10	17
45–49 "	24	16	40	13	7	20
50–54 "	15	8	23	8	3	11
55–59 "	8	3	11	6	1	7
60–64 "	7	7	14	3	5	8
65–69 "	4	4	8	2	1	3
70–74 "	3	2	5	2	1	3
75–79 "	0	1	1	0	1	1
80–84 "	1	1	2	0	0	0
85 years and over.	..	..	..	..	..	..
Totals.....	316	233	549	118	93	211

Among those *dying* from epidemic encephalitis in New York City in 1920 the probability that any individual picked at random would be a male was

$$p = \frac{118}{211} = .5592.$$

Whence

$$\frac{pq}{n} = .001168$$

$$p' = .4994 \text{ as before.}$$
$$p - p' = .0598 \pm .0230.$$

From this result we may conclude that the proportion of males dying from epidemic encephalitis in New York in 1920 was not significantly different from that found in the general population.

It thus appears that more males were attacked but no more died from encephalitis than would have happened if sex had no differential effect whatever relative to this disease. This

result can only mean that the case fatality rate is different in the two sexes.

Influence of Age.—It is stated in (2) that "No age seems exempt, but the greatest proportion of cases occurred in young adults, with a preponderance of males." This raises the following question: Is the age distribution of each of (a) male cases, (b) female cases, (c) male deaths, and (d) female deaths, significantly different, as a whole, from (e) the male population age distribution and (f) the female population age distribution?

Using the χ^2 method, and taking the 1910 age distribution of the New York City population as the basis of comparison, one gets:

For male *cases:*

$$\chi^2 = 8.254.$$
$$P = .410.$$

Or, one would expect in 4 out of every 10 trials with samples of the size here dealt with to get a divergence as great as or greater than that between the male case incidence age distribution and the general population age distribution, if epidemic encephalitis were perfectly random in its age incidence. In other words, there is slight basis for assuming that this disease attacks males more frequently at one age than another.

If we leave out the "under 5" group

$$\chi^2 = 4.111.$$
$$P = .767.$$

This means that in males 5 years old and over the age incidence of cases is very close indeed to a random one. In 77 out of every 100 trials there would result from chance alone deviations as great as or greater than that actually shown between male cases and the general male population.

Turning to male *deaths* we have:

$$\chi^2 = 18.450.$$
$$P = .018.$$

These values indicate a significant divergence of the male death age distribution from the general population male age distribution. But a further analysis gives:

For male deaths under 45 years of age:

$$\chi^2 = 5.790.$$
$$P = .448.$$

For male deaths under 35 years of age:

$$\chi^2 = 3.319.$$
$$P = .652.$$

It thus appears that it is chiefly the excess of male deaths beyond age 45 from this disease that causes the low value of P for the entire male death distribution.

Putting all the facts together it can be asserted, on the basis of the experience of New York City in 1920 with epidemic encephalitis, that in males the disease is probably not significantly more likely to attack one age than another, but instead falls upon males in about the proportion that they are normally present in the general population. It is furthermore not more fatal among the attacked at one age than another until after age 35 is passed. From that point on there are more fatal cases than would be expected if the toll of death were taken purely at random in respect of age.

Turning to the age distribution of female *cases,* and again comparing with the age distribution of the total female population (as of 1910) we get:

$$\chi^2 = 4.847.$$
$$P = .773.$$

It is thus quite certain that, so far as the experience of New York City in 1920 may be taken as typical, epidemic encephalitis occurs among females without definite age preference, striking the different ages in due proportion as they are represented in the general population. Females of any particular age are not more likely to contract the disease than females of any other age.

The age distribution of *deaths* among females presents a different case. It will be well to examine the facts in some detail. Table 3 gives the data.

TABLE 3.—DATA FOR TESTING THE DEVIATION OF THE FEMALE DEATH AGE DISTRIBUTION FROM THE FEMALE POPULATION AGE DISTRIBUTION

Ages	m Age distribution of female population (as of 1910)	m' Age distribution of females dying of encephalitis lethargica in 1920	$\frac{(m-m')^2}{m}$
Under 5 years.....	9.810	8	.3340
5– 9 years	8.553	2	5.0207
10–14 "	8.268	5	1.2917
15–19 "	9.427	5	2.0790
20–24 "	10.954	11	.0002
25–34 "	17.495	22	1.1600
35–44 "	13.044	21	4.8526
45–64 "	12.554	16	.9459
65 years and over..	2.894	3	.0039
Totals	92.999	93	15.6880

$$\chi^2 = 15.6880$$
$$P = .048.$$

It thus appears that the death distribution as a whole is significantly divergent from what would happen if the disease was as likely to be fatal at any given age as at any other. But it is also apparent that more than half of the large value of χ^2 is due to the contribution of but two age groups. These age groups are the 5–9 and the 35–44. The deaths in the former were fewer than expected and more than expected in the latter. The numbers are small, however, and it will probably be found, when more data are available, that the facts as to age distribution of deaths are the same in females as in males, namely that there is a definite tendency towards greater fatality in the higher age groups.

SUMMARY

It is distinctly to be understood that the results herein set forth have only such degree of validity as inheres in the experience of New York City with epidemic encephalitis during the two calendar years 1919 and 1920. Doubtless as more material accumulates new statistical relations of this disease will appear. But at the present moment the data here discussed are the most extensive in existence, so far as the writer knows. They fairly represent, in short, about all the basis of a statistical character which exists for forming an intelligent opinion *now* about the statistical features of this potentially important disease. The conclusions reached are, of course, subject to modification by more extensive experience.

What these conclusions are may be summarily stated as follows:

1. In the year 1920 the case incidence of epidemic encephalitis increased in New York City nearly five-fold over 1919. At the same time the case fatality rate increased from 26 per cent of the attacked to 37 per cent of the attacked. It is believed that this increase in case fatality rate is a real phenomenon, and it is certainly statistically significant.

2. The seasonal incidence, as judged by monthly distribution of cases was significantly different in 1920 to what it was in 1919.

3. The peak of mortality in an epidemic outbreak may be expected to occur from 23 to 37 days *after* the peak of case incidence (morbidity).

4. There is a significantly larger proportion of males among the attacked than there is in the general population, or, put in another way, males are especially susceptible, to a statistically significant degree.

5. Deaths occur among males no more frequently in comparison with females than would be expected from the normal proportions of the two sexes in the population at large.

6. The disease is not more likely to attack either males or females at one age than at another. The age distribution of attacked cases, in other words, does not significantly differ in either sex from the age distribution of the general population.

7. The age distribution of deaths does differ significantly in both sexes from the age distribution of the population. There appears to be a definite tendency for the disease to be more fatal in the higher age groups.

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THE THERAPEUTIC SIGNIFICANCE OF THE GRAM REACTION

By JOHN W. CHURCHMAN

Attention was called in 1912,[1] and in a number of publications which appeared in the following years, to a striking parallelism which exists between the gentian violet reaction and the Gram reaction. If organisms whose growth is inhibited by gentian violet be spoken of as gentian positive, and those whose growth is uninfluenced by the dye as gentian negative (Figs. 1 and 2), it is found that the large majority of Gram-positive organisms are gentian positive, while the large majority of Gram-negative organisms are gentian negative. The actual figures are as follows:

		Growth inhibited	Growth uninfluenced
130 species	Gram-positive 77	70 (90%)	7 (9%)
	Gram-negative 53	8 (15%)	45 (84.9%)
318 strains	Gram-positive 182	165 (95%)	17 (9.3%)
	Gram-negative 136	15 (11%)	121 (88.9%)

[1] Jour. of Exp. Med., 1912, XVI, No. 2, p. 221.

Moreover, it is just the organisms which are constantly and definitely Gram-positive (like *B. subtilis*) which are constant in their failure to grow even in the presence of minute quantities of the dye; and just the organisms which are constantly Gram-negative (like *B. coli*) which grow readily, even in fairly strong dilutions of the dye. (See Figs. 1 and 2.)

Rather striking, too, is the difference in avidity of the living Gram-positive and Gram-negative organisms for the stain, when examined in the hanging drop: *B. subtilis* rapidly becomes deep violet and promptly loses its motility, while *B. coli* stains slowly—some of the individuals very slowly—only a small proportion of the individuals stain at all deeply, and all retain their motility for some time. The criticism that (since the Gram reaction is a phenomenon exhibited by dead bacteria, while the gentian reaction is one exhibited by living bacteria) to stress the parallelism between the two reactions is unduly to magnify properties exhibited by living matter, on the one hand, and dead matter, on the other, was met by per-

forming the Gram stain on living organisms centrifugated in the test tube: The parallelism was again found to hold.

Facts of this sort made it natural to seek an explanation for the parallelism between Gram and gentian reactions in the assumption that both depended on the presence in the Gram-positive organisms, and the absence from the Gram-negative organisms, of chemical groups avid for certain chemical groups in the molecule of the stain. Saturation of these groups (Ehrlich's "fixation") would, according to this theory, lead to retention of the dye by the Gram-positives and their death when exposed to it; absence of these groups from the Gram-negatives would account for their incapacity to retain the dye by Gram's method and their ability to grow in its presence. This supposition received added support from the further observation that the selective bacteriostatic power was possessed not only by gentian violet but also by other members of the tri-phenyl-methane group: both *the dyes* belonging to this series, and *simpler substances* with similar molecular structure (Fig. 5). Evidence has, however, gradually accumulated which proves this hypothesis to be incorrect.

(1) In the first place, the parallelism between Gram reaction and gentian reaction—while striking—is not complete. There is a small but definite number of species in each group which do not follow the rule (Figs. 3 and 4).

(2) More important than this is the observation of a Gram-negative strain occurring in nature, which, although all other strains of the same species followed the rule and grew well in the presence of gentian violet, would not grow when the dye was present in the media on which it was planted.[2] This strain of *B. enteritidis* is shown in Fig. 6, stroked on a divided gentian violet plate along with the other four strains of the same species studied. Its exceptional behavior will be clear at a glance. Furthermore, it was not transient. It was observed in the two samples of this strain examined. Both samples were descendants of a strain isolated in Cambridge ten years before, and had been kept growing by hundreds of transplants in two different laboratories. Yet both had retained this remarkable inherent susceptibility to gentian violet which by no possibility could have been acquired by struggle against the dye. This strain of *B. enteritidis* was identical, by all morphological, tinctorial and cultural tests, with the other strains studied; it differed from them only in its growth-behavior toward gentian violet; it was as definitely Gram-negative as the other strains. This strain may be referred to as a "Strain-within-a-species" variant.

(3) It has recently been possible to isolate from a "pure" culture of *B. coli* a "strain-within-a-strain" variant which will not grow in the presence of gentian violet.[3] I have repeatedly made the statement that the growth of a Gram-negative organism, such as *B. coli*, proceeds on the gentian side of a divided plate as luxuriantly as it does on the plain agar. Such a statement would certainly seem to be warranted by an experiment like the one represented in Fig. 2, where *B. coli* has run wild over the gentian violet agar (as though it actually found the dye a growth accelerator), while *B. subtilis*, as usual, shrinks from it.

Further investigation of this subject, however, by quantitative methods, demonstrates that, if the experimental conditions be altered as regards the number of bacteria transplanted, the statements as to the effect of the dye on Gram-negative organisms must be considerably modified. If a divided plate be stroked with a 24-hour broth culture or a heavy suspension of *B. coli*, growth is equally luxuriant on the two sides of the plate (Fig. 7A). If, however, increasingly weak suspensions of the organism be used for the stroking, fewer and fewer colonies appear on the gentian violet agar and at high dilutions of bacterial suspension none at all may appear (Fig. 7, B and C). By separate cultivation of the colonies which appear on the plain agar side of such plate it has been possible to isolate from a "pure" culture of *B. coli* a gentian-positive and a gentian-negative strain. These two strains, labelled for convenience strain *X* and strain *Y*, show their difference in behavior toward gentian violet, whether they are stained and then planted on plain agar or are stroked across divided gentian violet plates. This specific characteristic was not transient, for the experiment illustrated in Figs. 8 and 9 was done after the two strains had been allowed to grow for six weeks through a large number of transplants, and it will be seen that the original features of the two strains have been preserved. Cultural studies of these two strains showed them to be identical in every respect. These facts may be interpreted as follows:

There may exist within a single bacterial strain two types of individuals which, although in every other morphological, tinctorial and cultural characteristic identical, are quite dissimilar in their reaction toward gentian violet, one growing vigorously and the other not at all on media containing this dye. The latter we may refer to as a "strain-within-a-strain" variant.

No observations of variants, which at all approach these in nicety of selective behavior toward bacteriostatic agents, have hitherto been made in the bacterial field. One may speak of the phenomenon presented as reversed fastness, inasmuch as unlike the usual type of fastness seen in the protozoan field in which it is the ordinary run of organisms which are susceptible and the occasional organism which is fast, in this instance it is the ordinary run of organisms which are fast and the occasional organism which is susceptible.[4]

The variant strains of *B. enteritidis* and *B. coli* just referred to are definitely Gram-negative. *The factor which determines the reaction of an organism toward the Gram stain is not necessarily, therefore, the same as the factor which determines its cultural behavior toward gentian violet; and the chemical affinity hypothesis must be abandoned as the sole explanation of the parallelism between Gram and gentian reactions.*

[2] Jour. of Exp. Med., 1912, XVI, No. 6, p. 822.

[3] Jour. of Exp. Med., 1921, XXXIII, No. 5, p. 569.

[4] It is interesting to note that, after the report of the variant type of *B. enteritidis* and of the gentian-positive strain of *B. coli* just described, De Kruif has reported as an instance of microbic dissociation the isolation, from a single culture of *B. lepisepticum* of two strains, one of which was virulent and the other avirulent.

FIG. 1.—Divided gentian violet plate stroked with Gram-positive and Gram-negative organisms to show that the growth of the former is prevented by the dye and the growth of the latter uninfluenced. *C, B. coli; O, Oïdium albicans; A, M. aureus; T, B. typhosus.*

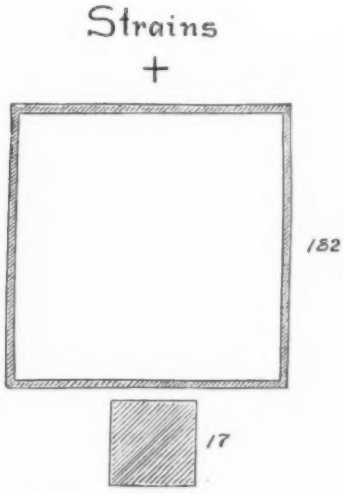


FIG. 3.—Schematic representation of effect of gentian violet on Gram-positive organisms; study of 182 strains. The central white area represents the number inhibited by the dye (165), the shaded border the number unaffected (17). The area of the shaded border is shown in the shaded square.

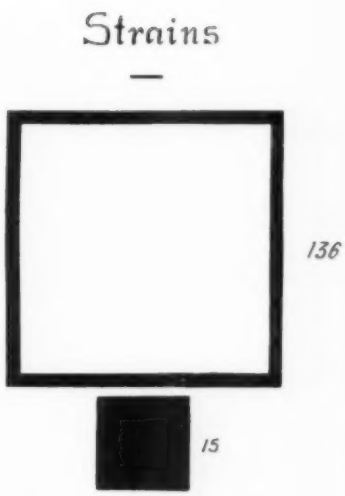


FIG. 4.—Schematic representation of effect of gentian violet on Gram-negative organisms; study of 136 strains. The central white area represents the number unaffected by the dye (121); the black rim the number killed (15). The area of the black border is shown in the black square. It will be noted that the facts are just the reverse of those for the Gram-positives.

FIG. 2.—Note that the Gram-negative *B. coli* (*C*) has spread wildly over the gentian agar, while the Gram-positive *B. subtilis* (*S*) shrinks from it.

FIG. 5.—Experiment to show that a dye chemically related to gentian violet (Dahlia) possesses a similar selective bacteriostatic power. *C, B. coli; A, B. anthracis; S, M. aureus; T, B. typhosus.*

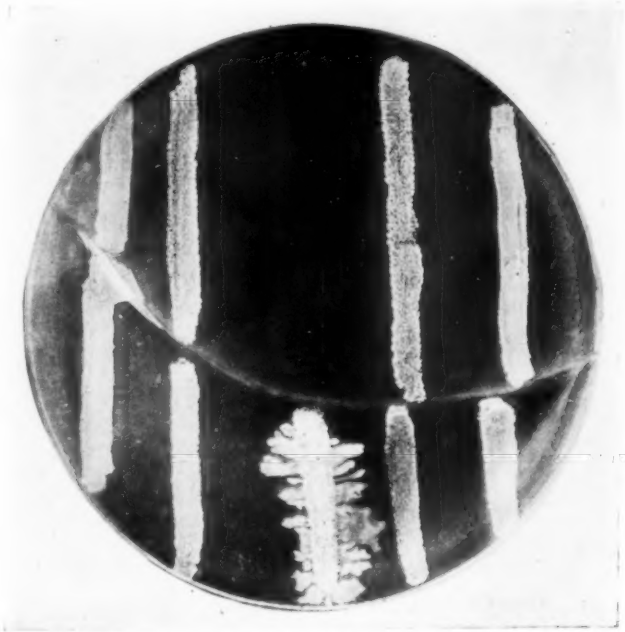


FIG. 6.—Divided gentian violet plate, showing a strain-within-a-species variant. Five strains of *B. enteritidis* have been planted; all except one have grown on the gentian violet agar.

FIG. 8.—Divided gentian violet plate stroked with two strains isolated from a single "pure" culture of *B. coli*. The growth of strain *X* is uninfluenced by the dye; that of strain *Y* is entirely prevented. Strain *Y* is referred to as a strain-within-a-strain variant. The two strains are identical by all other tests.

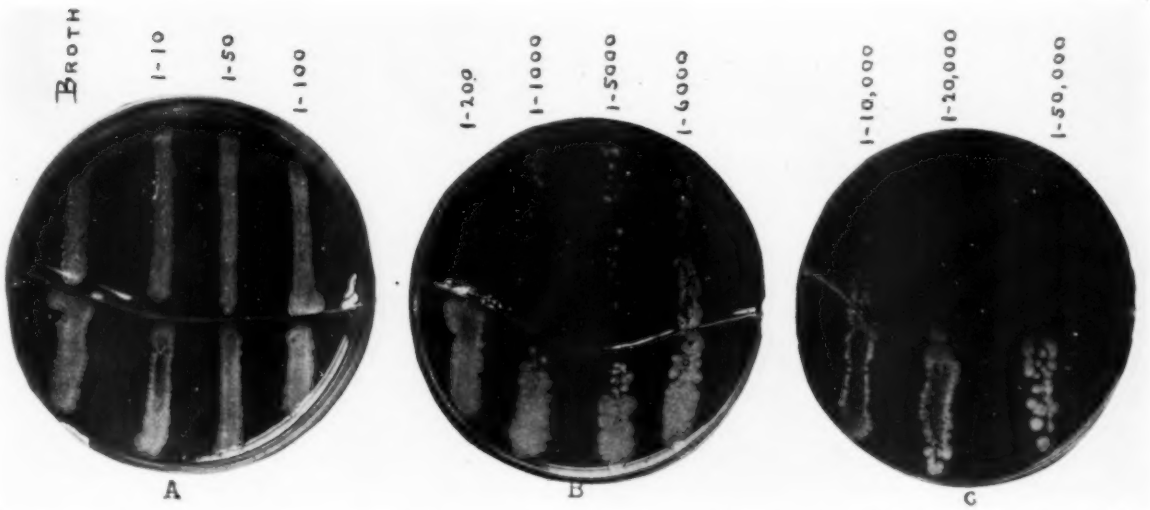


FIG. 7.—Divided gentian violet plates, stroked with increasingly weak bacterial suspensions. Note that in Plate A growth is equally good on the two sides of the plate; but that as the bacterial suspension becomes thinner the colonies become fewer on the gentian violet agar and finally disappear entirely (plates B and C). Compare with Figs. 1 and 2.

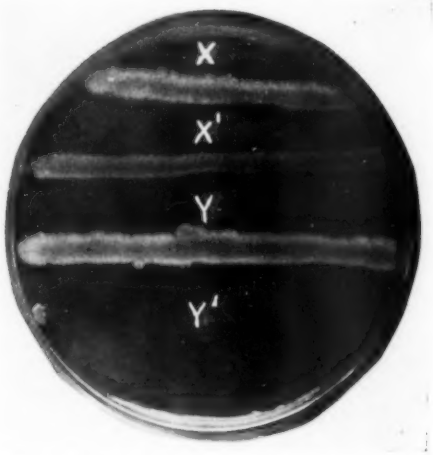


FIG. 9.—Plain agar plate stroked with strains *X* and *Y* to show the effect on them of exposure to gentian violet. *X*, Strain *X* unstained for control. *X'*, Strain *X* which has been stained with gen. vi.; *Y*, Strain *Y* unstained for control; *Y'*, Strain *Y* which has been stained with gen. vi.

FIG. 12.—Persistence of a Gram-negative organism in a wound treated with gentian violet. The organism obtained from the wound has been stroked across the plate at the sides (*C*). *A* is a stroke of *M. aureus*, for contrast.

FIG. 10.—Experiment to show that a dye chemically unrelated to the tri-phenyl-methanes may also possess the selective bacteriostatic power. Upper half of the plate contains agar to which bismarck brown has been added; this is an azo-dye. *C, B. coli; T, B. typhosus; S, B. subtilis; A, aureus.*

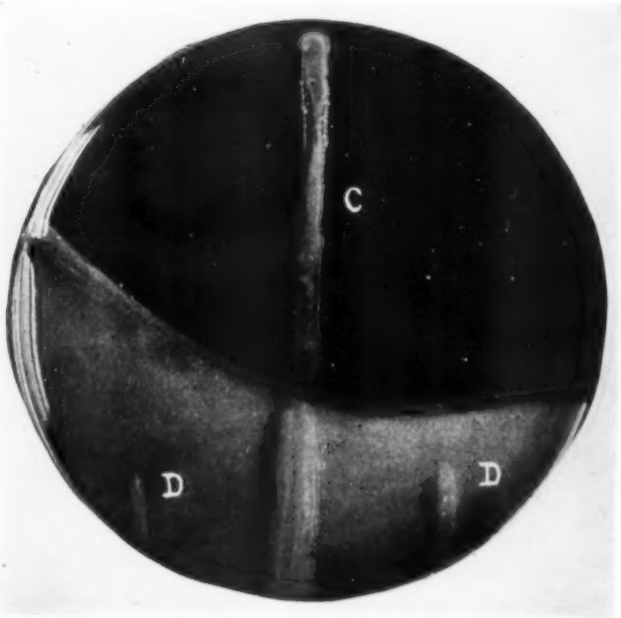
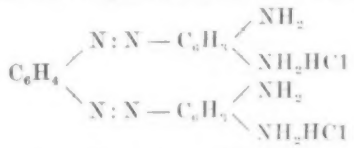


FIG. 11.—Effect of gentian violet on *B. diphtheriæ*. Divided plate stroked with this organism (*D*), and with *B. coli* (*C*) for contrast.

(4) But there are other considerations which warrant the belief that the chemical affinity hypothesis is not the correct one, or at least that chemical affinity is not the only factor involved. It was at first believed that the selective bacteriostatic power manifested by gentian violet and other members of the tri-phenyl-methane series was peculiar to this group. Most members of the group examined exhibited it (Fig. 5), while most of the dyes belonging to other chemical groups did not. The experience with bismarck brown, however, showed that in drawing the conclusion that the selective power was limited to the tri-phenyl-methane group, we had fallen into an error which the previous experiments had given no cause to suspect. This dye does not belong to the tri-phenyl-methane series. It is an azo-compound and has the formula:



In the early experiments there had been no evidence that it possessed any selective bacteriostatic power. It was found later, however, that, by varying the amount of bismarck brown used in the media, a dilution could be reached at which this dye, too, prevented the growth of Gram-positives, while without effect on the Gram-negatives (Fig. 10).

Although the fact has never been brought out by those who have written on the bactericidal power of the flavines, it is probable that these dyes too are less effective against the Gram-negative organisms than they are against the Gram-positives. In one of the few careful bacteriological studies, made during the war, of wounds which had been treated with these dyes Bashford[5] mentions with surprise the fact that, although in wounds treated with Dakin's solution it is the cocci which disappear last, in wounds treated with flavine the commonest organism to outlive others on the wound surface is a Gram-negative bacillus.

(5) A recent study by Kämmerer[6] has brought still more convincing evidence against the hypothesis of specific affinity between chemical group in the tri-phenyl-methane molecule and the Gram-positive organisms to account for the selective activity of these substances. This observer has shown that an exactly similar selective bacteriostatic power is possessed by mesohematin and certain metallic compounds of mesoporphyrin (*e. g.*, compounds with manganese and magnesium).

Mesohematin, when added in very weak solutions to the media on which organisms have been planted, inhibits the Gram-positive organisms but is without effect on the growth of the Gram-negatives. This substance has the formula $C_{34}H_{36}O_4N_4FeOH$, and is quite unrelated structurally to the aniline dyes.

The ultimate explanation of the Gram reaction is not yet known, but the trend of present experimental study is in the direction of a mechanical rather than a chemical theory of the process. Kämmerer regards the greater susceptibility of Gram-positive organisms to mesohematin and to gentian violet as due to their greater permeability. In his opinion these substances really reach the interior of the positive organisms and thus kill them; the Gram-negatives they are only able to attack on the surface and they therefore do them no harm. For this conception there is a large amount of evidence. It is, however, somewhat difficult to make my own experiments with stained small groups (containing 30 individuals) of *B. coli* conform to it. These organisms were exposed to the dye under microscopic observation until they had become stained a deep black. It was hard to imagine that the dye was simply resting on the surface of the bacteria. Yet they grew luxuriantly when planted on agar.

While the mechanism of the Gram reaction, therefore, though probably not chemical in nature, is still the subject of some discussion, the important point which the gentian violet studies have brought out is that it is of clinical significance. Speaking generally the Gram-negative organisms are less susceptible to many, possibly to all, selective bacteriostatic substances than the Gram-positive organisms. General protoplasmic poisons will kill Gram-positives and Gram-negatives alike. But these will also kill, or greatly injure, tissue cells. Substances which act in selective fashion will be more likely to be effective against the Gram-positives than against the Gram-negatives; hence, the latter will be the more resistant organisms. To this rule there will be exceptions presented by the very delicate Gram-negatives, like the gonococcus, whose growth may be hindered by a variety of slightly unfavorable conditions in the environment; and by the very vigorous Gram-positives, like some of the spore-bearing anaërobes whose growth is inhibited only by strong solutions of gentian violet.

A study of attempts to sterilize infected amputation stumps with gentian violet emphasized the significance of the Gram reaction of the infecting organism, at least so far as gentian violet is concerned. This study has been reported elsewhere.[7] Stumps infected with the Gram-positive *B. diphtheriæ* were rather easily freed of this organism; those infected with the Gram-negative *B. coli* could not be sterilized with gentian violet (Figs. 11 and 12).

[5] British Med. J., 1917, II, 849.

[6] Arch. f. Exp. Path. u. Pharm., LXXXVIII, Hft. 5 and 6, S. 247.

[7] Jour. Am. Med. Assn., 1920, Jan. 12, vol. 74, pp. 145-148.

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THE TREATMENT OF PLACENTA PRÆVIA, TOGETHER WITH THE ANATOMICAL DESCRIPTION OF TWO SPECIMENS

By William B. Thompson

(From the Obstetrical Department of The Johns Hopkins Hospital and University)

During the past few years a rapidly growing mass of literature has accumulated which advocates the adoption of more radical methods in the treatment of placenta prævia. Particularly since the appearance of Krönig's article upon the subject the employment of Cæsarean section has been recommended as the safest and most conservative method of treating selected cases of this serious complication of pregnancy and labor, and certain writers have even gone so far as to advocate its routine employment.

For the past decade, at least, Dr. Williams and his associates have maintained and put in practice the belief that except in rare instances conservative measures give better results so far as the mother is concerned than more radical operative interference. In order to determine whether the results obtained justify such a belief, I have gone over the first 10,000 admissions to the obstetrical ward of The Johns Hopkins Hospital and have analyzed the cases of placenta prævia included therein. I shall take this occasion to summarize the results of my investigation, and afterwards I shall describe in detail the anatomical findings in two specimens obtained from patients in the series, which will be illustrated by drawings kindly made by Mr. Max Broedel.

TABLE I.—SHOWING INCIDENCE OF PLACENTA PRÆVIA

	Admissions	Number of prævia	Percentage of prævia
Full term deliveries	7,752	33	50
Premature deliveries	635	21	32
Duration of pregnancy not stated.	3	3	4.5
Abortions	651	8	12
Died before delivery	15	1	1.5
Discharged before delivery	657	..	..
Admitted post partum	208	..	..
Not pregnant	79	..	..
Total	10,000	66	100

Table I shows that 10,000 women were admitted to the ward from its opening in 1896 to July 31, 1919, of whom 9054 were delivered at or before full term.

In the series 66 cases of placenta prævia were noted—an incidence of 1 to 137, or 0.73 per cent. Such a figure, however, gives too high an idea of the frequency of the complication; for during the same period an approximately equal number of patients were cared for in the out-patient service, and were sent into the hospital for treatment, whenever serious complications arose. Accordingly, our figures must be divided by two, which gives an incidence of 1 to 274, or 0.37 per cent. Even this figure is still too high, as many of the patients were not originally cared for in the service, but were referred to it after the midwife or physician in attendance had recognized the seriousness of the condition, as well as their inability to treat it properly. Taking these facts into consideration it would seem conservative to estimate that placenta prævia does not occur oftener than once in 400 pregnant women, and therefore may be regarded as a comparatively rare complication.

While it may be noted from Table I that 50 per cent of the cases occurred in full-time deliveries, closer consideration of the figures will show that the complication occurs relatively more frequently under other conditions. Thus, upon taking into consideration only the patients admitted to the ward, it is found that in full term deliveries the incidence was 1 to 235, or 0.43 per cent, as compared with 1 to 30, or 3.31 per cent, at premature labor. In other words, our figures indicate that placenta prævia occurs nearly eight times more frequently at premature than at full time labor, although it must be admitted that such an estimate is only approximately correct.

In view of the relatively large number of colored patients in the service, it may be interesting to ascertain whether the racial factor appears to have any influence upon the incidence of placenta prævia, and Table II gives information upon this point, as well as upon the influence exerted by the multiparity of the patients.

TABLE II.—SHOWING INFLUENCE OF RACE AND MULTIPARITY

	White	Black	Total		White	Black	Total
1—para	6	2	8	10—para	1	2	3
2—para	6	1	7	11—para	1	0	1
3—para	6	3	9	12—para	1	0	1
4—para	6	2	8	13—para	1	1	2
5—para	7	1	8	16—para	1	0	1
6—para	5	2	7	17—para	1	0	1
7—para	3	0	3	Not stated	2	1	3
8—para	0	1	1	Total	48 or 72.7%	18 or 27.3%	66
9—para	2	1	3				

As approximately 45 per cent of the patients admitted to the service are black, one should expect, roughly speaking, that a similar proportion of placenta prævia cases should be noted in that race. Yet, our figures indicate that the incidence is

only about two thirds of the calculated expectancy. Whether this discrepancy is to be attributed to the relatively small number of cases with which we have to deal, and is therefore only apparent, or whether it is due to some definitely racial influence cannot be decided until more extensive observations are available. A point which speaks in favor of the latter view is that if multiparity and the existence of endometritis play the part usually assigned to them in the etiology of placenta prævia that complication should occur more frequently in the black than in the white race, on account of the unusual prevalence of such conditions in the former. As our figures, however, show that placenta prævia occurs less frequently, a certain presumption is raised in favor of some racial influence—a point to which attention has not previously been directed.

Table II likewise demonstrates the influence of repeated child-bearing in the production of the abnormality, and abundantly confirms the well known observation that primiparæ are much less liable to placenta prævia than multiparæ. As forty per cent of our patients enter the service to have their first child, approximately 26 of our 66 placenta prævia patients should have been primiparæ, but our figures show that only eight of them were.

Analysis of the figures also shows that the multiparous women had on the average a much larger number of children within a short time than normal. Thus, the 55 multiparæ concerned averaged 5.9 children each in 9.7 years from the date of the first delivery, which greatly exceeds the fecundity of the average married woman, and lends further support to the generally accepted view that rapidly recurring pregnancies favor the development of disturbances of the endometrium, which in turn predispose towards abnormal implantation of the placenta.

TABLE III.—SHOWING THE AGE OF THE PATIENTS

Age	White	Black	Total
16–20 years	4	5	9
21–25 years	11	3	14
26–30 years	11	3	14
31–35 years	9	5	14
36–40 years	9	0	9
41 years on	2	1	3
Not stated	2	1	3
Total	48	18	66

Table III analyzes the ages of the patients when treated for placenta prævia. Naturally, it brings out the fact that the complication occurs most frequently during the most active period of child bearing. Possibly, the only two points of interest shown are that a comparatively large number of women present the abnormality before they have passed their twentieth year, and, on the contrary, that very few women near the menopause are involved. It does not, however, seem permissible to attempt to draw any definite conclusions from so relatively few cases.

Table IV gives the relative frequency with which the various types of placenta prævia were noted in the series, as well as the percentage incidence of each, and also indicates the number of maternal deaths. Our figures are in accord with

TABLE IV.—SHOWING THE FREQUENCY OF THE SEVERAL TYPES OF PLACENTA PRÆVIA AND THE MATERNAL AND FŒTAL MORTALITY IN EACH

Type	No. cases	Percentage	Maternal deaths	Fœtal deaths
Centralis	14	21.21%	4	13
Partialis	27	40.91%	2	21
Marginalis	24	36.36%	0	13
Lateralis	1	1.52%	1	1
Total	66	100.%	7	48

those of most writers upon the subject and show that partial placenta prævia is the most usual variety, while the central type occurs much less frequently. Attention should be directed to a relative decrease in the number of centralis cases diagnosticated during recent years. This is not the result of any actual diminution in the number of such cases, but is due to greater accuracy in diagnosis. It would appear that in the early years of the service the diagnosis of placenta prævia centralis was sometimes based upon finding the region of the internal os entirely covered by placental tissue when the cervix was only sufficiently dilated to permit the introduction of one or two fingers. Further experience has demonstrated, however, that a diagnosis made at that time is not always correct, as it has sometimes happened in such cases that examination after the cervix had become fully dilated has shown that it was only partially covered by placental tissue, so that what had appeared as a central placenta prævia at the first examination was in reality only a partial one.

Stress should be laid upon this point, and the diagnosis of the central type should not be made unless the cervix is fully dilated, or, unless the finger introduced through the partially dilated internal os can feel placental tissue extending sufficiently far beyond its periphery to insure that it will still cover it when dilatation is complete. Such a restriction is particularly necessary at this time, for the reason that not a few writers advocate very radical treatment in the central type of implantation.

As was indicated in Table IV, seven of the 66 mothers were lost—a mortality of 10.6 per cent. At first glance this seems abnormally high, but when allowance is made for the fact that a large proportion of the women were not admitted until late in labor and after profuse hæmorrhage and extensive manipulation, the results, while leaving something to be desired, are not bad.

The complication was treated by various methods, and while the number of cases is too small to permit the drawing of absolute conclusions, the experience of the service, nevertheless, definitely indicates that some procedures are much safer than others. Table V gives a synopsis of the methods of treatment

TABLE V.—SHOWING METHODS OF TREATMENT

Method of treatment	Cases	Maternal deaths
a. Died undelivered	1	1
b. Braxton Hicks' version	2	1
c. Rupture of membranes	2	0
d. Cæsarean section	2	1
e. Admitted in 2d stage	8	2
f. Manual dilatation of cervix	15	2
g. Ballon	36	0
Total	66	7

employed, together with the number of maternal deaths associated with each. Each method will be considered briefly, after which an attempt will be made to decide which is the safest and the most dangerous, respectively.

The case of the patient who died undelivered will be described in more detail at the end of the article (Case I), and is of especial interest, as her death was the only one in the series following the use of the ballon. Moreover, it is particularly interesting, because the excellent drawing accompanying the description gives graphic evidence of how slight a degree of placental separation may lead to fatal hæmorrhage.

The two patients who were treated by Braxton Hicks' bipolar version followed by slow extraction presented the partial and marginal type of placenta prævia, respectively. One died as the result of excessive loss of blood, and the other recovered. Such results are entirely accidental and do not give a fair idea of the merits of the procedure, which in general should be regarded as the best treatment when rubber ballons are not available.

In two other patients with marginal placenta prævia, rupture of the membranes sufficed to check further separation of the placenta, while prompt engagement of the presenting part controlled the bleeding and allowed labor to progress spontaneously. Doubtless this method of treatment, which is so popular in France, might have been employed advantageously in other marginalis cases.

The two instances in which Cæsarean section was employed require especial mention. Both were examples of complete placenta prævia. The first occurred in a 40-year-old primipara, who was also suffering from chronic nephritis, while the second constituted an accidental finding in a uterus which was removed by supravaginal amputation on account of atresia of the cervix and intra-partum infection. The latter will be described in detail as Case II, and the excellent drawing accompanying it will afford an ideal illustration of a complete placenta prævia *in situ*.

In the first patient, the condition was complicated by a rigid primiparous cervix and an inelastic pelvic floor, so that, although the child was dead at the time of admission, it was felt that Cæsarean section offered the most conservative method of delivery. The patient died on the eighth day, and autopsy showed that death had resulted not from infection, but from an exacerbation of the nephritis from which she had suffered for years. This case is of further interest, as it represents the only one in the series which afforded Dr. Williams and his associates an indication for treating placenta prævia by this means. Naturally, a single experience cannot be utilized as an argument either for or against any method of treatment, but the case may be utilized as showing how rarely one encounters what is regarded here as a valid indication for the employment of Cæsarean section—the association of a rigid cervix with placenta prævia.

Eight patients were admitted in the second stage of labor, and as dilatation of the cervix had already taken place spontaneously, all that remained was to effect delivery by the most conservative means available, such as forceps, version and extraction, etc. It is interesting to note that in three of these cases the child presented transversely, and in one of them the shoulder was so impacted and the uterus in such tetanic contraction that version was out of the question and delivery was effected after decapitation. This patient died, as well as one other, who was admitted after a long course of maltreatment outside. The latter presented a rhachitic pelvis which was so contracted that a Cæsarean section would have been imperatively indicated had she been in good condition: but as the child was dead and the mother was profoundly shocked as the result of loss of blood and fruitless attempts at delivery, and furthermore as signs of intra-partum infection were present, delivery by craniotomy was effected as the last resource; the patient, however, died shortly after her return to bed.

It must be admitted, I think, that in neither of these instances can the fatal issue be attributed to the underlying condition, nor to the treatment employed in the service, as both are examples of the type of neglected cases which unfortunately are met with in all clinics.

Passing on to the group of 15 cases in which delivery was effected after manual dilatation of the cervix, the following may be said. In the early years of the service *accouchement forcé* was the routine method of delivery whenever the cervix was not sufficiently dilated to permit prompt termination of labor by the ordinary obstetrical procedures. In such circumstances the cervix was dilated manually by Harris's method, after which delivery was effected by version and extraction, with the exception of a single case in which the existence of a primary breech presentation made version unnecessary.

In this group we had to deal with the following varieties of placenta prævia—6 centralis, 4 partialis and 5 marginalis—and 2 mothers and 11 children were lost. In one of the fatal cases, the patient was moribund on admission, and would

probably have died irrespective of the method of treatment, so that her death can scarcely be attributed to the means by which delivery was effected. On the other hand, the other death was directly attributable to the operation. In this instance, which was reported to the American Gynecological Society by Dr. Williams, in 1906, the markedly exsanguinated multiparous patient was admitted with complete placenta prævia and the cervix somewhat less than half dilated. While Dr. Williams was examining her, profuse hæmorrhage occurred and the cervix seemed so soft that he felt that it could readily be dilated. Manual dilatation was effected with the greatest ease, the child turned and extracted and the placenta expressed. The cervix was then inspected and a deep tear extending to the fornix was discovered on the left side. This was exposed and apparently satisfactorily repaired, the patient being put to bed in good condition. Without further external bleeding, she soon began to do badly and died four hours later. At autopsy it was found that the tear had involved the lower uterine segment, and that only its lower portion had been repaired by the vaginal sutures, with the result that the bleeding had continued between the folds of the left broad ligament and had led to the formation of a hematoma 15 to 18 cm. in diameter.

Although members of the staff had long realized that manual dilatation of the cervix in placenta prævia was usually accomplished at the expense of deep cervical tears, which frequently required immediate repair, the fatality just described still more forcibly accentuated the dangers of the procedure, and led to its abandonment as a routine practice, together with the recognition that generally speaking it represents the most dangerous method of treating this complication.

Following the recognition of the danger of *accouchement forcé*, the use of the rubber ballon has in recent years become the routine method of treating placenta prævia except when the cervix is fully dilated when the patient is admitted to the service. Prior to the World War the reinforced Champetier de Ribes' ballon was employed with the greatest satisfaction, but since that time we have been forced to use Vorhees' bags instead. These are of domestic manufacture, and leave a great deal to be desired, in that they are less efficient as dilating wedges, and are distinctly less durable.

Ballons have been employed in 37 cases, 36 of which will be considered under this heading, while the remaining one will be described below as Case I. In the series the following types of placenta prævia were noted—6 centralis, 17 partialis and 14 marginalis. All of these patients recovered.

Ordinarily, the ballon is introduced into the uterus after rupturing the membranes, or after perforating the placenta in centralis cases, in preference to the extraovular method advocated by Kosmak and others, because it has been felt that better results are obtained by compressing the placenta against the area of separation. Where haste was essential, traction was made upon the bag by means of a weight attached to the end of the tube and allowed to hang over the foot of the bed. Usually the largest-sized bag was allowed to remain in place until it was expelled spontaneously, after which the course pursued varied according to circumstances. If the bleeding had ceased, and the patient was in good condition spontaneous delivery was awaited, and occurred in 10 of the 36 cases. On the other hand, if the bleeding continued, or if the patient was in poor condition or if the uterine contractions appeared inefficient, delivery was effected by the most conservative means available, the records show that version and extraction were employed in 18, breech extraction in 5, and forceps in 3 cases.

More particularly since we have been compelled to employ Vorhees' bags, complete dilatation of the cervix has not always been effected, so that in 5 instances it has been necessary to complete the process by manual methods. These, however, are not comparable with the *accouchement forcé* which was formerly employed, since, as the major part of the dilatation had already been effected by the ballon, all that is required is to overcome the final resistance. Such secondary manual dilatation was required three times on account of premature and repeated rupture of the ballon, and twice on account of tetanic uterine contractions, or because the patient's condition was so serious that it was felt that she could not survive a longer delay.

As has already been indicated, all of the 36 patients in this series recovered, and such a result is the more remarkable, as they were not treated by a single individual possessing unusual dexterity and judgment, but by a succession of resident obstetricians who naturally varied considerably in ability. With such maternal results, it can justly be claimed that that treatment by means of the ballon is eminently satisfactory, and accordingly one can readily understand why the employment of Cæsarean section has found so little favor in this service.

Of course, it must be admitted that the latter affords a ready method of coping with the situation, and does not require the hours of waiting incident to more conservative methods of treatment. On the other hand, it must be borne in mind that many of the patients, who are admitted exsanguinated and after having been subjected to vaginal manipulations by physicians with imperfect technique, are poor surgical risks, so that the mortality following Cæsarean section in them must inevitably exceed that noted in our service. Furthermore, it should be remembered that the uterine cicatrix following Cæsarean section represents a *locus minoris resistentiæ*, so that the possibility of its rupture in subsequent pregnancies constitutes a danger, which certain writers consider so great as to justify the dictum "once a Cæsarean always a Cæsarean." Consequently, if a Cæsarean is done, it may condemn the patient to repeated sections in any future pregnancies. If this is the case, the unprejudiced observer must admit that it is a questionable procedure so to treat the ordinary case of placenta prævia that this will be necessary, more particularly as other methods of treatment are available, which give as good or even better immediate results and do not compromise the future child-bearing career of the patient.

On the other hand, it is argued by the advocates of radical treatment that Cæsarean section offers additional advantages in the way of better prospects for the child, and at first glance such an argument appears convincing, more particularly when

we admit that less than 30 per cent of the children in our service were saved. It should, however, be remembered that placenta prævia is a complication which tends to become manifest at a period when the child's prospects are minimal. Our figures show that it occurs eight times less frequently at term than at premature labor, so that we ordinarily have to deal with children which have no chance for continued existence after birth, or which are so premature that they readily succumb to the loss of blood or other conditions incident to the condition. Consequently, it seems justifiable to demand that Cæsarean section should be employed only under the strictest indications, and should not be considered as a routine method of treatment. In addition, it should be realized, that placenta prævia is inevitably associated with a high infantile mortality, and consequently obstetricians should hesitate to advocate methods of treatment which, while they may save the lives of a few more children, do so at the expense of increased maternal mortality.

I shall now briefly describe two cases in the series, from which we were fortunate enough to obtain, at autopsy, specimens which throw important light upon some of the anatomical features of placenta prævia, and whose value is greatly enhanced by the fact that Mr. Max Broedel was kind enough to prepare drawings illustrating certain salient points.

Case I.—H. 9795. The 21-year-old patient had previously had two spontaneous labors. She registered in the out-patient service and one week later had a severe hæmorrhage, but did not call the service until she was markedly exsanguinated. She was admitted to the hospital *in extremis*, and presented the characteristic symptoms of acute anæmia.

Examination showed that the pregnancy was practically at term and that feeble uterine contractions occurred from time to time. The external os was 3 to 4 cm. in diameter, with placental tissue covering the internal os. A large-sized bag was immediately introduced, a salt solution infusion begun, and various stimulants administered. Although not more than 200 c.c. of blood was lost, the patient died undelivered three hours after admission.

The ballon was removed after death and the autopsy gave negative results, except for the signs of severe secondary anæmia. The pregnant uterus was removed unopened and placed in formalin. After hardening, it was opened by a sagittal median section, when it was found that we had to deal with a placenta prævia partialis, which was inserted upon the posterior wall of the uterus. The placenta measured 16 x19 cm.; it varied in thickness from 1.5 cm. at its upper part to 3.5 cm. at its lower pole, and extended from the internal os two-thirds of the distance to the fundus. Fig. 1 shows that it was firmly attached to the uterine wall, except for a distance of 2 cm. just above the internal os, where it had been separated from its attachment, and it was from this area of detachment that the fatal hæmorrhage had occurred. The cervical canal was intact, measured 4 cm. in length, and presented the usual rugous appearance with a clear mucous secretion.

Examination of microscopic sections through the cervix showed no abnormality except a moderate leukocytic infiltration. Sections through the uterine wall and placenta above the area of separation showed that the placenta was typically implanted upon a thin decidua basalis; while sections through the area of separation showed that the detachment had occurred in the spongy layer, the greater part of the decidua being attached to the detached portion of the placenta, while only a very thin layer remained in contact with the muscularis. The decidua covering the detached pole was moderately infiltrated with leukocytes, particularly at its lowermost extremity. At the upper angle of the area of separation, a large vein had been torn through, and readily explains the origin of the fatal hæmorrhage.

This specimen is of interest from two points of view. It shows, in the first place, how small an area of detachment may lead to a fatal termination; while in the second place it represents the only fatality in the series following the use of the rubber ballon. As the patient was practically moribund when admitted, her death can not be ascribed to the treatment pursued, as the result would have been the same whatever might have been done.

Case II is of interest particularly because it affords an opportunity to study and portray a central placenta prævia *in situ*.

Case II.—H. 5381. The 35-year-old patient was sent to the hospital by her physician on April 27, 1912, with the statement that she had been in labor three days, had no cervix, and required operative delivery. The patient stated that she had had three spontaneous labors, the last six years previously, and one miscarriage two years later. Shortly after the miscarriage she was operated upon at another hospital when her cervix was amputated, the right ovary removed, and the uterus suspended. A year later she was operated upon at a second hospital when the right tube and part of the left ovary were removed, and numerous adhesions freed. At that time the uterus was found to be firmly fixed to the anterior abdominal wall, but the fixation ligament was not interfered with.

On admission to the hospital the temperature was 100.6° F. and the pulse 85. On examination the pelvis was found to be normal, the fundus reached midway between the umbilicus and ziphoid, and was so tetanically contracted that the outlines of the fœtus could not be distinguished. Fœtal heart sounds were not heard. A pigmented scar, 12 cm. long, occupied the midline of the lower abdomen. Palpation showed that the round ligaments diverged upwards, and that a softish flattened structure, the size of a finger, lay to the left of the abdominal scar and was thought to be adherent intestine. On vaginal examination no trace of the cervix could be found, but the vault of the vagina was occupied by a nodular area which was marked by several radiating depressions extending from a common center. Examination with a speculum gave similar results. In view of the condition of the cervix radical interference seemed necessary, and it was debated whether this should consist in Cæsarean section or in a vaginal incision through the cervical region. In view of the existence of intra-partum infection and the fact that the child was already dead, it was determined to remove the unopened uterus by deep supravaginal amputation. At the same time it was recognized that the operation would probably be complicated by the fixation of the uterus, as well as by intestinal adhesions.

After the usual preparation the old scar was excised and the abdominal cautiously opened in the midline. The adherent intestine, which had previously been palpated, extended parallel to the incision for a distance of 6 to 8 cm. on the left side. A second loop of intestine was likewise adherent to the abdominal wall and extended at a right angle across the incision. This was peeled off and the incision was extended above the umbilicus. The elongated fixation ligament then came into view and was represented by a band 4 mm. in diameter extending from just below the lower extremity of the abdominal incision to the anterior surface of the uterus just beneath the fundus. After this had been doubly ligated, the uterus was eviscerated when it was found that the right ovary was absent, while the corresponding tube was 4 to 5 cm. in length and presented a patent fimbriated extremity. The left tube, which was converted into a hydrosalpinx, was densely adherent to the uterus, as well as to a loop of intestine at its distal end.

The uterus was then amputated unopened as low down through the cervix as possible. This was comparatively difficult, owing to the fact that the lower uterine segment was distended by a bulky portion of the fœtus. In the center of the cervical stump a small conical depres-

FIG. 1.—Sagittal section through lower and posterior portion of the uterus and cervix from a patient dying undelivered (×⅓). It shows a partial placenta prævia in situ, and demonstrates how small an area of separation may lead to fatal hemorrhage.

FIG. 2.—Sagittal section through a full term pregnant uterus removed unopened by supravaginal amputation (×⅓). It shows a central placenta prævia *in situ* with velamentous insertion of the umbilical cord. The tag of tissue on the upper part of the anterior wall represents the proximal end of the false ligament resulting from the previous fixation. Note the cup-like form of the placenta, and its variations in thickness in different locations.

sion, apparently lined with mucous membrane, was noted, which was excised by a funnel-shaped incision. The stump was closed by interrupted catgut sutures, after which the broad ligament wounds were united in the usual manner. The adherent loops of intestine were then freed from the abdominal wall and the incision closed in four layers. None of the uterine contents escaped into the abdomen, and very little blood was lost. The recovery was very satisfactory, the highest temperature being 101.5° F. on the tenth day, and the patient was discharged on the twenty-fourth day. At that time the cervix was represented by a few nodulations in the vaginal vault, but no trace of the external os could be discovered.

Description of Specimen.—Immediately after removal, the uterus was placed in formalin, and after hardening formed a pyriform mass, 26 x 22 x 18 cm. In the midline of its anterior surface, 6 cm. below the fundus, is the uterine end of the fixation ligament, while the uterine attachments of the round ligaments are at a slightly higher level and are 19 cm. apart. The right tube is apparently normal, except for a glistening cystic structure, 1.5 cm. in diameter, on its anterior surface; the left tube is covered by adhesions. The left ovarian ligament is so thinned out as to resemble a peritoneal fold, and at its free end is an elongated mass of tissue, 2.6 x 6 x 7 cm., which probably represents the ovary.

At the site of the cervical amputation is an opening, 6 cm. in diameter, which is occupied by dark spongy tissue resembling the placenta in appearance. This is firmly adherent posteriorly and to the right, but is free anteriorly and to the left. Upon opening the uterus by a sagittal median incision a large macerated male child was found in the right acromio-dorso-anterior position, the left shoulder being firmly impacted in the lowermost part of the uterus. Upon removing the child, it was found that we had to deal with a placenta prævia centralis, which filled out the entire uterine segment like a cup. It measured 8.5 cm. on the posterior and 12 cm. on the anterior wall of the uterus, and was much thicker posteriorly than anteriorly, measuring 1.5 and 0.7 cm., respectively. Corresponding to the line of amputation through the cervix the apex of the placental cup is very thin and does not exceed 3 mm. in thickness. Coursing over the inner surface of the anterior portion of the placenta are many large vessels, which extend upward to join the cord (Fig. 2).

Figure 2 shows very clearly that the major portion of the placenta is firmly united to the uterine walls, except for a distance of 5 cm. on the anterior and 4 cm. on the posterior wall of the lower uterine segment, where a small space exists. The umbilical cord is inserted velamentously upon the anterior wall of the uterus 2 cm. above the upper margin of the placenta and large vessels make their way downward through the membranes toward the latter. Generally speaking the placenta appears smaller than the average, and on section presents a pale dense appearance so that, except for a small portion, it seems to be devoid of blood.

The uterine walls vary from 11 to 3 mm. in thickness, being thinnest in the lower uterine segment. There is no indication of a contraction ring, and the protrusion noted on the posterior wall probably resulted from pressure against the promontory of the sacrum.

Microscopic Examination.—Sections through the uterine wall above the placental site show that the amniotic epithelium has become desquamated and that the chorionic membrane is represented by a layer of clear connective tissue from which the nuclei have almost entirely disappeared. The decidua vera is in great part normal. It is made up of small decidual cells and contains a few flattened glands. At its junction with the chorionic membrane there are numerous areas of closely packed polymorphonuclear leukocytes in various stages of preservation, which apparently represent minute miliary abscesses. The muscularis is normal and its fibers in great part follow a parallel course.

Sections through the posterior wall at the placental site show that the placenta is almost entirely infarcted and presents numerous areas of calcification. No trace of decidua is seen beneath it, for the reason that the tissue is so densely infiltrated with leukocytes as to obscure all details of structure. The leukocytes extend far out into the muscularis, forming parallel rows between its fibers. Sections through the upper part of the placental site on the anterior wall show an identical appearance, but lower down the placenta is separated from the anterior wall of the uterus by a narrow space. The free surface of the placenta is covered by a layer of tissue which is so densely infiltrated with leukocytes that it is impossible to determine its histological character; while the corresponding uterine wall is acutely inflamed and the leukocytic infiltration extends through the entire thickness of the muscle, and has so obliterated all structure that it is impossible to say whether its internal surface had been covered by decidua or by cervical mucosa, although its corrugated appearance and the presence of a few irregular glandular spaces make it probable that we have to deal with the latter. Furthermore, the free portion of the placenta below the site of implantation is covered by a membrane which is so acutely inflamed as to obscure all details of structure.

This specimen is of great interest from three points of view. First, as to the origin of the atresia; second, the mode by which the uterine infection occurred; and third, the abnormal implantation of the placenta. Naturally, the first thought would be that the atresia had followed the high amputation of the cervix four years previously, but the fact that the patient had continued to menstruate regularly up to the onset of the present pregnancy clearly indicates that the complete atresia could not have existed; moreover, if it had, impregnation could not have occurred.

Concerning the origin of the uterine infection, it can only be said, that as the inflammatory reaction is so intense and involves the entire interior of the uterus, it is improbable that it is of recent origin. For this reason, it seems permissible to assume that a moderate atresia had followed the operation, but had not been sufficiently pronounced to prevent the escape of the menstrual discharge or to interfere with conception, and that some time after the occurrence of pregnancy the patient had attempted to induce abortion; that this had not had the desired result, but had led to inflammatory changes, which resulted in the complete closure of the atresia and the infection of the interior of the uterus. Against such a supposition is the fact that the patient positively denied any such attempt.

The velamentous insertion of the cord permits speculation as to the cause of the abnormal implantation of the placenta. From what we know of the ordinary processes of implantation, it may be assumed that the point of origin of the uterine end of the cord corresponds to the point of attachment of the abdominal pedicle in early pregnancy, which in turn corresponds to the portion of the chorion which later forms the placenta. For this reason it may be assumed that the ovum was originally implanted upon the middle of the anterior wall of the uterus, but for some reason the chorion frondosum found the tissues in that location little adapted for implantation and consequently the placenta was developed lower down, and, as sufficient room was not available for it on the anterior surface of the uterus, it extended over the internal os to the posterior surface, thus giving rise to the prævial condition.

ON THE SUPPOSED LIFE-CYCLE OF BACTERIA*

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Most diseases from which mankind suffers are due to parasites, many of which, for instance, Treponema pallidum, under natural conditions, are unable to live outside the human body. On the other hand, some of them have an independent life, as saprophytes or parasites outside of the body, and only occasionally become pathogenic. Bacillus tetani and actinomyces may be cited as examples of this kind. A third group of pathogenic organisms have a regular life-cycle, so that a part of their development takes place inside, another part outside the human body. The malarial organism is the classical paradigm for this group.

Concerning bacteria, the general consensus of opinion has been that they have no life-cycle of this kind. Their morphology has been considered to be relatively simple. But during the last decade many scientists have attacked this problem from different points of view and reached conclusions differing from the usual opinion. The first to emphasize this idea against the popular theory was Ernst Almquist, who as early as 1884 pointed out, in disagreement with Koch, that the epidemiology of typhoid fever could not be explained on the assumption that the virus was produced only in the human body. According to Almquist, the typhoid bacillus develops a saprophytic stage outside the body, during which it not only propagates but even acquires new and important characteristics.

The basis for these ideas was that the theory adopted by Koch could not explain why the epidemics appeared at certain times of the year and did not account for the peculiarities shown by the curve of morbidity during epidemics caused by infection through the water supply.

In this connection, you can recall some other peculiär epidemiological facts hitherto unexplained, for instance, the changing virulence during different epidemics, or the problem how in some diseases—influenza, for example—the pathogenic organism survives from one epidemic to another. Now, if it is possible that a life-cycle takes place outside the human body, it may be considered likely that such may occur in the body itself. The difficulty in demonstrating the specific organism in certain disturbances caused by syphilis or tuberculosis has brought many to the idea that the microörganisms are here in a special unknown stage of development. Important records that could support the theory of a cyclic development of certain bacteria inside the human body are given by Hort and Mellon. I shall refer to these later.

It might be best first to consider the original investigations of Almquist in order to understand better the somewhat discordant results of later authors on this subject. According to Almquist, the typhoid bacillus grown on drying agar at low temperature does not propagate in the usual way by simple division but in the following special way. Coccoid bodies are developed from the rods by budding. These coccoid bodies are of two kinds: first, irregular protoplasmatic masses, and, second, sharply defined round ones with thick walls. From both kinds, rods or filaments can grow later and they in their turn produce new coccoid bodies. The different cells are kept together for a long while by fine connecting threads. The filaments can be very numerous and form an intricate system, which was called by Almquist the "myceloid." To the protoplasmatic masses he gave the name "bacteria plasmodia." Regarding the thick-walled bodies, Almquist made the surprising statement that they contained several separate small cells that were liberated by the bursting of the shell.

These bodies which bud from rods or coccoids, Almquist called exogenic bodies or bacterial conidia, but did not describe the more particular connection between the different forms. Those developed at low temperature had no ability to move and some of them showed an increased virulence, while those exposed to high temperature lost their virulence.

The question of virulence was taken up for further study by the collaborators of Almquist—Korén and Olsson, who primarily used Löffler's bacillus and Vibrio choleræ for their investigations. The first of these organisms when grown on a special medium at low temperature produces small needles measuring one or two microns. These are avirulent, but when transferred to the usual media they regress to the normal form of the bacillus, regaining their virulence.

The investigations of Olsson on Vibrio choleræ are more elaborate. He found that the vibrios, when grown for a longer time on soil extract, lost their ability to move and grew longer in shape. When these were transferred to agar, they produced shorter, still motionless, and less virulent forms. These peculiarities were visible through a number of generations, but after a while the vibrios returned to the original normal form, but showed a toxicity up to ten times stronger than in the original cultures.

Other observations made by Almquist are worth mentioning. In filtering about a hundred typhoid cultures he found that in ten of these there was to be seen a very small coccoid microorganism, which was most difficult to grow and which was entirely different from the typhoid bacillus. At the same time, however, this aberrant form was agglutinated by typhoid serum in a dilution of 1 to 500 and produced, when injected into rabbits, antiserum for typhoid. Almquist suggested that this form might be a "mutation" and because of the fact that it produced antiserum for typhoid he has given it the peculiar name Bacterium antityphosum. Almquist found other small immovable forms by examining blood from typhoid patients in the hanging drop. He makes no attempt to explain these different phenomena in referring the bacteria to other groups of microörganisms. He only remarks that the plasmodia remind him of similar structures in the myxomycetes

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and that the conidia are not unlike the bodies of this same name in the group of the fungi.

The problem of the systematic position of the bacteria was early considered by Sopp. He holds the opinion that the bacteria are really a kind of fungi. His view has been supported by A. Meyer, who in a long series of papers has demonstrated the striking resemblance between the structure of the cells in these two groups. During the four years in which I have been occupied in studying problems of this kind I have been brought to hold a similar view, that the bacteria are *fungi imperfecti*. I shall first try to show how far such a theory explains the above-mentioned facts discovered by Almquist and others. What are then the characteristics of such a fungus? Take as an example a monilia, and you will see that it propagates in two different ways—by budding and by simple fission. The buds can develop into short coccoids, to longer bodies like bacilli or to long filaments. The budding can occur at any point of the cell. The development of branches is substantially the same as in a building. On the other hand, both the short buds and the long filament can produce new cells by fission. If it is a filament that falls into a row of cells we speak of oïdia, and when buds arise on the side of a filament we call them conidia.

Sometimes you will find only one of these forms in a culture and sometimes several mixed together, according to the nature of the substrate and the age of the culture. If, for instance, you grow the uusual Oïdium albicans on agar you will only get coccoid forms, while you will find long forms and filaments if the culture is transferred to Löffler's serum. The filamental forms here become more and more pronounced during the series of generations. Investigations have shown that all these different forms can be found among nearly every kind of bacterium. If they do not appear on the usual substrates it is only necessary to change the chemical composition of the substrate or the temperature of the incubator. Striking pictures of these aberrant forms are to be found in the papers of Almquist, Sopp, De Negri, Hort, Meirowsky, Löhnis and Smith, Mellon, Wade and Manalang, Jones, Bergstrand, and others. The photographs given by Hort of bacteria of the typhoid group, and those of Treponema pallidum in the papers of Meirowsky are very clear.

Anyone can easily control these points by examining, for example, the Löffler's bacillus in the hanging drop. But it is absolutely necessary to avoid using dried and stained slides, for in a preparation like this every kind of artefact may interfere.

The objection that all these different forms depend upon contaminations is readily refuted, inasmuch as several of the authors named have used warm-stage methods and followed the development of the different cells. Furthermore, the forms in question occur in all cultures. To speak of contamination would mean to say that no pure cultures of bacteria exist. For this reason we need not consider the methods of Burri and Barber in making cultures from single cells.

In one of my papers I have given a review of the literature concerning different groups of Coccaceæ, Bacteriaceæ and Spirillaceæ, and have pointed out that they all behave in the same way with the exception of the spore-producing Bacteriaceæ which show an extraordinarily simple and uniform morphology.

Someone may ask if it would even be possible to grow staphylococci or meningococci so that they will change their form to rods. This is not possible—at least not at present—but Korén and Hort have demonstrated that they can reproduce by budding and this is the essential thing. In this connection compare the behavior of the different kinds of bacteria with that of various types of fungi. Saccharomyces is generally very stable and can only with difficulty be forced to change its form on various substrates, whereas Oïdium albicans can easily be grown as coccoid bodies or as filaments.

It is impossible in this short paper to compare in any detail the structure of the cells. This has been dealt with to a certain extent in some of my previous papers. To serve as an example from this field, I shall merely call attention to the fact that the polar granules of Löffler's bacillus are placed in a vacuole where they exhibit a dancing movement in just the same way as " die Tanzkörper " in the yeast cells. The theory will also probably throw light on such questions as Much's granules and the fragmentation spores of Boström.

So far it is easy to interpret the different forms of the bacteria by comparing them with fungi, but there are other forms that present considerable difficulties.

As we have said, Almquist found spherical forms of different kinds among typhoid bacilli. Most of these are probably just the usual vegetative cells, " short buds "; but others were thick-walled and these are apparently of another kind. These have been seen by quite a number of observers. In the diphtheria bacillus these walls show the characteristic that they can be stained with the Ziehl-Nielsen stain, and one would be likely to compare these with the durable cells as we find them in yeast. I have advocated this explanation and thus agree completely with Jones, who, in speaking of azotobacter, writes as follows:

> In cultures more than fourteen days old, large spherical, thick-walled cells are common. These appear to be resting cells or arthrospores. On transference to fresh media these thick-walled cells germinate, the cell plasma emerging from the thick wall as a large rod, which at once proceeds to multiply by fission.

Mellon's suggestion that these forms are possibly in some way connected with a rejuvenescence of the protoplasm must not be left out of consideration. Such processes are described by Woodruff in protozoa in which pure strains that are prevented from conjugation rejuvenate regularly by an intracellular process called endomixis. Even real conjugation, as in Saccharomyces, is said by Löhnis and Smith to occur among bacteria. These ideas may seem somewhat daring, but it is in fact most natural to look for some kind of rejuvenescence like that which occurs in other microörganisms.

Interesting and rather startling observations concerning these thick-walled cells have been published by Almquist, Hort and Mellon. They claim that these bodies contain within their walls a number of small cells that become free by bursting

of the wall. Mellon has seen these small cells move freely after liberation. Except for the mobility of the small cells there is here a striking resemblance to the sporulation of yeast cells, especially since the number of small cells within the wall seems to be fixed. In pictures shown to me by Mellon four small cells could be seen within each body. The supposed mobility of the small cells reminds one, on the other hand, of certain forms of protozoa. The importance of this mobility must not be exaggerated. We have seen that Bacillus typhosus and Vibrio choleræ can occur in mobile and immobile forms at the time when they show a typical fungus growth.

Jones has made somewhat similar discoveries working with Bacillus azotobacter. The only difference is that Jones did not observe the small cells inside spherical thick-walled bodies but within bodies of other shape. He writes:

> Following a period of reproduction by fission, individual cells develop reproductive granules or gonidia within their cell plasma, which on disintegration of the mother cell are dispersed, increase in size, become typical azotobacter short rods, ovals or spheres, and reproduce by fission.

We shall now consider the question of the nature of the bacteria plasmodia as described by Almquist, and the "symplastic stage" from the records of Löhnis and Smith, two probably corresponding features which we occasionally find mentioned also in very early literature. The two latter authors, especially, have considered this stage most important as the beginning and end of the life-cycle of bacteria. I can offer no suggestion as to the nature of these forms, whose importance also is advocated by Jones. I will only mention that I have seen similar structures in Löffler's bacillus when using India ink preparations, but little was to be seen in the hanging drop and stained slides; only gentian violet made such structures visible. But this stain is peculiar in that it stains the mucus that almost always occurs around old bacteria, so that it is difficult to recognize the shape and form of the bacterium itself. I was inclined to interpret these masses as clusters of degenerated bacteria kept together by mucus. Of course, it cannot be denied that in these masses there may be forms that are capable of reproduction.

It is not difficult to imagine that toxicity and virulence could be changed during such a supposed life-cycle, although it is not necessary to use such a life-cycle as an explanation for such changes. As Oïdium albicans shows such different morphological forms, depending upon the nature of the substrate, why should not changing environmental influences cause modifications in the physiological behavior of the organism? The investigations of Olsson on Vibrio choleræ show just this. Information given me by Rettger concerning experiences in his laboratory points in the same direction. This must not be taken to mean that a certain toxicity must be connected with a certain morphological form. Morphological and physiological characters are both changeable although not necessarily in a parallel way.

We shall now approach the question as to the possibility of filtering bacteria. The theory that bacteria are fungi imperfecti allows of two different possibilities: First, that a bud or a supposed endospore is so small that it is able to pass the filter; or second, that a piece of plasma containing nuclear substance can be sufficient to propagate the cell, as is the case with certain algæ. Attempts made by Almquist, by Löhnis and Smith, and by Jones, to filter the bacteria and obtain a new culture from the filtrate have not been followed with success. The only investigators that have succeeded in this undertaking are Hort and Mellon. The latter obtained from blood filtrate an invisible microörganism that developed into a polymorphous bacterium, and the former injected filtrates of spinal fluid from cases of meningitis into monkeys and obtained from the urine on agar among other forms the meningococcus (Weichselbaum) and diplococcus (Jaeger). By cultures Hort has been able to raise "giant meningococci," which watched on the warm stage show endosporulation and budding. In this connection, he writes:

> The meningococcus of Weichselbaum is not a bacterium. It is an ascospore derived from the "giant meningococcus" by a process of endosporulation. The filterable meningococcal virus probably represents a stage in the life-cycle of the ascomycetic organism described.

Hort considers the filterable form of importance in the spreading of meningitis, and a similar supposition concerning Pfeiffer's bacillus and influenza is held by Wade and Manalang, who found that this organism shows a fungus growth. Furthermore, Hort claims to have isolated from cases of typhus a very small bacillus that is polymorphous and pathogenic for monkeys. From these animals he later obtained the same forms, besides larger bacteria which he considers other developmental forms of the same organism. Considering the great possibility of contamination, it seems safe to regard all these cases with a certain amount of scepticism until they have been controlled on a larger scale. It seems to me not unlikely, however, that the typhus bacillus is a polymorphous bacterium. Pictures representing Ricketsia show a diplococcus-like microörganism whose two parts are connected with a thin filament. Such diplococcus-like forms are usually accompanied by rods and filaments. I should not be surprised if large portions of the group which Prowazek calls Chlamydozoa should turn out to be polymorphous bacteria.

In conclusion, a few words concerning what we call bacterial mutations. Most of the papers on this subject are of very limited value, inasmuch as they do not consider the normal variability of the bacteria. Where there is the possibility that a real mutation did occur, the question of contamination always raises doubt as to the interpretation of the observed facts. Almquist's bacillus antityphosus could possibly be explained as something similar to the phenomenon of d'Herelle, especially since Salimbeni has shown it to be probable that this phenomenon may be due to a parasitic organism living on B. Shiga-Kruse.

How much of this that I have presented is true, and how much of it is false, is at present impossible to say. But, at any rate, it seems that a great and promising field for investigation has been laid open. We must not forget that such groups as bacteria, protozoa and fungi are artificial divisions

made by us for our convenience. They have to be corrected as our knowledge develops.

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ERRATUM

Attention is called to an error on page 199 of the June BULLETIN in the article entitled "An Atypical Bacillus Paratyphosus B Infection" by Dr. Hugh J. Morgan. Table I in the left hand column of page 199 should be inserted between paragraphs 1 and 2 in the right hand column of page 199. See following arrangement:

At this time the patient's serum agglutinated a killed culture of B. paratyphosus B in dilutions of 1:50. There was no agglutination with B. paratyphosus A.

The low titer of our antiparatyphosus B serum against the unknown, together with the knowledge of the fact that organisms apparently falling into the paratyphosus B group are notoriously deceptive as to their identity, indicated the necessity of employing other methods for its identification. Castellani's absorption method was used. For purposes of simplification the organism under discussion will be called Bacillus "X" throughout the remainder of this report.

TABLE I

"A. T." Serum *	Before absorption	After absorption with Bacillus "X"
v.		
B. paratyphosus B (N. Y.)	Agglutination 1–2,500	Agglutination 1–2,500
"A. T." Serum	Before absorption	After absorption with B. paratyphosus B (N. Y.)
v.		
Bacillus "X"	Agglutination 1–1,000	No agglutination

*A polyvalent antityphoid serum containing agglutinins for B. typhosus, B. paratyphosus A and B. paratyphosus B.

Thus it was shown that Bacillus "X," while agglutinated by the specific paratyphosus B fraction of the "A. T." serum used, was unable to remove the paratyphosus B agglutinins from the serum.

THE CAPACITY FOR PHAGOCYTOSIS SHOWN BY POLYMORPHONUCLEAR LEUCOCYTES IN DEAD ANIMALS AND AFTER PRESERVATION IN SALT SOLUTION

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One of the disturbing surprises at autopsy is the occasional presence of leucocytes containing bacteria in transudates that have repeatedly been reported as sterile. This can in some instances, of course, be explained by assuming that bacterial invasion and phagocytosis have occurred during the agonal period. In many of the recorded cases, however, the clinical examinations and cultures have been made so recently before death that the remaining time is altogether inadequate to account for the extensive phagocytosis observed. Indeed, under some conditions the only admissible explanation for the presence of intracellular organisms is that the invasion and phagocytosis have taken place after the death of the patient.

Two opinions are generally entertained as to the significance of the occurrence of bacteria within phagocytes in the tissues and exudates of an infected animal after its death. The more common supposition which arises from this finding at autopsy is that the microorganisms were ingested by the phagocytes while the animal was yet alive. On this basis attempts are made to estimate the degree of resistance which the animal opposed against the infecting bacteria, and the phagocytosis is considered as an evidence in favor of the assumption that the microorganisms were etiologically important in the disease from which the animal suffered. While undoubtedly, in most instances, these assumptions are true, they should be weighed against the possibility that the phagocytosis observed at autopsy has occurred after the death of the animal.

The record in the literature of the continued activity of phagocytes independently of the functional coordination of the animal of which they were a part, is still fragmentary and incomplete. It, therefore, seems worth while to supplement this record with some disconnected observations on the viability of leucocytes made during the course of an experimental investigation of phagocytosis, which is being conducted in this laboratory.

There are a few recorded observations in the literature on the extension of the life of phagocytic cells after separation from the animal body. In a photographic study of the activity of the white cells of the blood Commandon [1] found that the leucocytes in blood preparations remained alive for 10 days if kept at a temperature of 10° C. When kept at 25° C., they continued alive for about 24 hours. However, when he attempted to preserve them at 38° C., their movements ceased and they died very soon. In 1915 Kobzarenko [2] noted that he was unable to find in the literature any indications on the capacity of leucocytes for independent existence. Accordingly he conducted a series of experiments and found that about 90 per cent of the leucocytes kept at incubator temperature were dead before the end of 48 hours. Approximately 50 per cent died during the first 24 hours. Weinberg and Sequin,[3] during an investigation of the inhibitory effect of hydatid liquid on phagocytosis, found that phagocytes immersed in this fluid were still capable of engulfing bacteria at the end of one hour. Hollande and Beauverie [4] demonstrated that leucocytes arising from a urethral exudate remained alive in the urine and exhibited phagocytic powers for as long as 10 hours, provided albumin were present. Wollmann [5] in an experiment of a somewhat different nature records that he kept leucocytes alive for 12 days within a collodion sac which was enclosed in the peritoneal cavity of a guinea-pig. Rowley [6] described a case of anemia in which the blood phagocytes, after being separated from the body for three months, were still capable of engulfing red cells.

In the above experiments wherever there has been any considerable extension of the life of the leucocytes after separation from the body, it has been accomplished by keeping them in the medium in which they occurred in the body. Then, after separation, the cell suspensions were kept at a constant temperature. Any alteration in the medium or marked change in the temperature seems invariably to have been accompanied with the most disastrous results.

The observations made in this paper were upon polymorphonuclear leucocytes and other cells subjected to the most varied circumstances. While the suspensions were sometimes kept in the refrigerator, no other precaution was observed to preserve the cells, and no special effort made to prolong their life, other than providing a trace of serum to prevent autolysis.

A sterile peritoneal leucocytosis was induced in each of four guinea-pigs by the injection of 5. c. c. of a suspension of aleuronat in physiological salt solution containing 3 per cent starch. Fifteen hours was selected as the time providing the maximum number of cells. These four animals were then subjected to the experiments described below.

Guinea-pig No. 1.—This animal was killed and left in the laboratory at room temperature for two hours. It was then injected intraperitoneally with a suspension of B. proteus. After one hour, samples of the exudate were withdrawn and examined. Many of the leucocytes had taken up the bacteria and were rapidly digesting them. Specimens of the exudate taken before the injection of organisms were culturally sterile and repeated smears did not reveal the presence of a single bacterium, intra- or extra-cellular. The microbes were taken up two to three hours after the death of the animal and in many cases were completely digested, as indicated by resolution into granules and loss of staining characteristics. Cells A and B in the accompanying plate illustrate the appearance of two representative phagocytes from this exudate.

Guinea-pig No. 2.—This guinea-pig was killed and after the removal of the abdominal viscera the animal was placed in the refrigerator and left there for seven days. At the conclusion of this time the peritoneum was washed out with salt solution, the resulting cells sedimented and a sample incubated with sensitized B. proteus for one-half hour. The most energetic phagocytosis ensued. Fifty

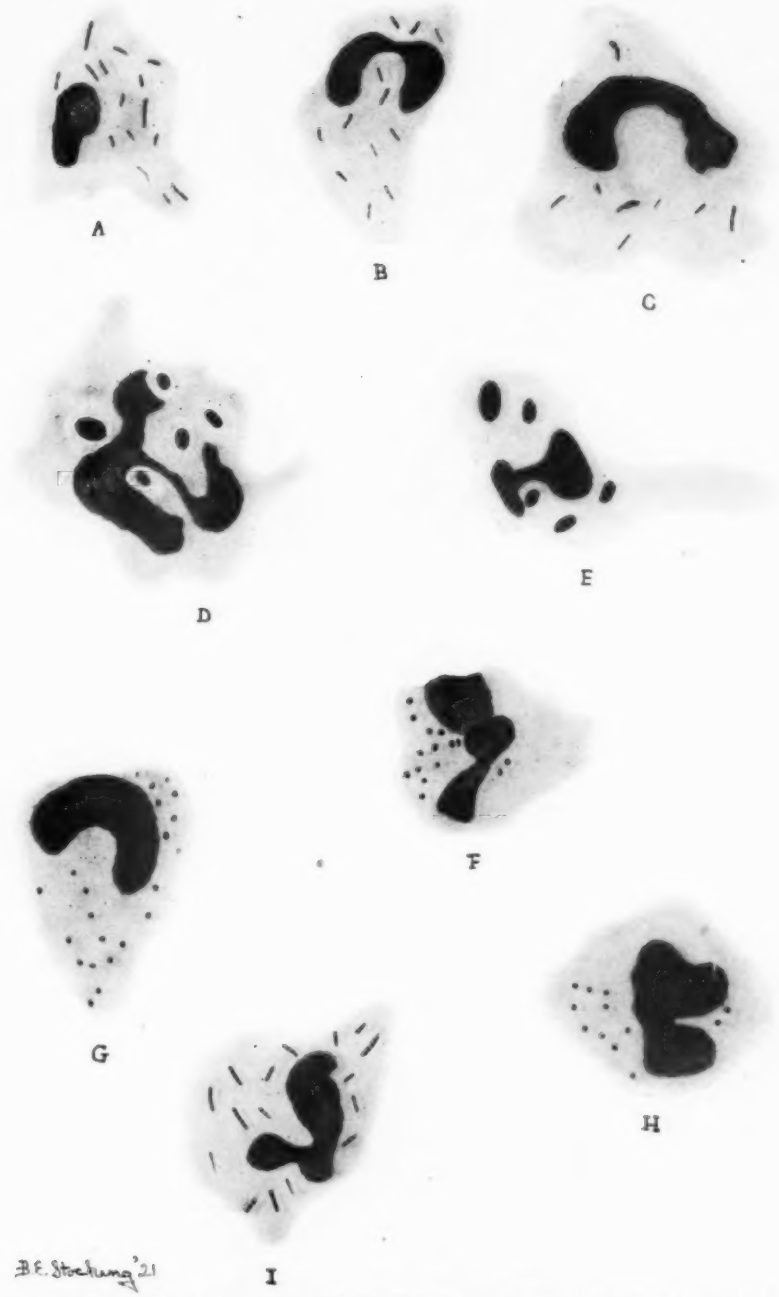

FIG. 1.—Camera lucida drawings (×1200) illustrating the appearance of phagocytic cells described in this paper. Leucocytes *A* and *B* represent an example of post-mortem phagocytosis. *C* is a polymorphonuclear leucocyte which, after 11 days in the dead body, was still actively motile and capable of phagocyting bacteria. *D* and *E*, leucocytes from the pleural cavity of a dog, remained active eight days after the death of the animal. The ingested yeast cells are surrounded by digestive vacuoles. Leucocytes *F*, *G*, and *H*, obtained from the peritoneal exudate of a guinea-pig, represent active phagocytosis after preservation in physiological salt solution for 18 days.

polymorphonuclear leucocytes counted contained more than 200 bacilli. Many of the cells were completely disintegrated, but others were apparently undisturbed and stained characteristically. The complete absence of bacteria in the original material was satisfactorily demonstrated.

GUINEA-PIG No. 3.—The peritoneal exudate was removed from this animal under sterile conditions and suspended in eight volumes of citrated salt solution (0.85% salt and 1.5% sodium citrate) and kept for six days. Four hours of each day the cells were kept at room temperature, four hours in the incubator, and the remaining time in a refrigerator with a temperature between 8° and 15°. Samples were removed each day and incubated with unsensitized bacteria. The results are given in the accompanying table.

Day	Number of cells	Serum	Number of Org.
1	50	None	125
2	50	None	135
3	50	None	121
4	50	None	160
5	50	None	120
6	50	None	00
	50	½ vol.	500

The exudate was slightly hæmorrhagic and the presence of the extravagated serum probably accounts for the continued spontaneous phagocytosis. The experiment was discontinued on account of a contamination.

GUINEA-PIG No. 4.—The exudate from this animal was removed sterile and suspended in fifty volumes of citrated salt solution. The cells were kept for 18 days, part of the time at room temperature and the remaining time in the refrigerator. On incubation with sensitized staphylococci the first day the polymorphonuclear leucocytes ingested an average of seven bacteria each. A similar incubation on the third day resulted in a phagocytic index of five. On the eighteenth day only a few of the leucocytes were active and intact but phagocytosis was still evident and some cells took up as many as 12 cocci. Sketches F, G and H are made from three of these phagocytes.

A guinea-pig, which had died in the laboratory as a result of a pleural hæmorrhage after cardiac puncture, was placed in the refrigerator and left there for 11 days. At the conclusion of this time some of the fluid from the pleural cavity was withdrawn and on examination was found to be free of bacteria and to contain numbers of apparently normal polymorphonuclear leucocytes. A quantity of this material was incubated with sensitized B. coli. At the end of half an hour almost all the whole leucocytes in the smears contained from one to ten bacteria. The complete absence of organisms from the original material was conclusively established. An inspection of 100 cells did not reveal the presence of a single microbe after the incubation, whereas almost every polymorphonuclear leucocyte contained bacilli. Sketch C illustrates the appearance of a cell from these smears.

A dog died in the laboratory and at autopsy showed a pleural effusion containing a few unusually large polymorphonuclear cells. The animal was kept for three days at a temperature a few degrees above zero. Some of the fluid was then removed and kept in the laboratory for five days. The leucocytes had remained 72 hours in the body following death, and then, after being kept five additional days in a test-tube, were still actively motile and energetically ingested sensitized bacteria. Sketches D and E were made of phagocytes from this series.

A sample of citrated dog's blood was taken and left standing at room temperature for several hours. The sedimented cells were then placed in the refrigerator for four days. They were then removed and placed in the incubator for several hours. At the conclusion of this time a quantity of the leucocytes was incubated with sensitized bacteria, with a resulting energetic phagocytosis. Fifty cells counted contained 250 bacteria.

While there has been no opportunity or wish to subject human phagocytes to the rigid experimental conditions described above, the following data would seem to indicate that these cells too are capable of sustained independent existence and that they retain their functional capacities, and may reveal them under the most astounding circumstances.

The urine from a patient suffering with chronic urethritis was collected and left standing on the table for three hours. The sedimented cells were washed once, suspended in physiological salt solution and then placed in the ice-box for 24 hours. They were again placed on the table for one hour. The cells were next incubated for 15 minutes with sensitized bacteria. Enumeration revealed a phagocytic index of 17. (Sketch I.)

Polymorphonuclear leucocytes derived from this source have since been kept in this laboratory for as long as three days and when brought in contact with sensitized bacteria have revealed a phagocytic index only slightly lower than that obtained with freshly isolated cells. Indeed, it appears certain that the phagocytic index is influenced more often by the manipulation and medium than by the age of the cells.

The conditions recited above are, of course, exceptional. It is not always possible to duplicate these observations at will, and, indeed, it is often quite impossible except by accident. These results do, however, indicate that polymorphonuclear phagocytes possess to an extraordinary degree the capacity for independent existence, and when kept under appropriate conditions, removal from the body does not entrain, for a time at least, any demonstrable impairment of normal function.

DISCUSSION

It is possible that some may object to the instances of unusual phagocytosis presented in this paper, and insist that what has been interpreted as phagocytosis might better be explained by assuming that the cells and bacteria have been superimposed in smearing. Such hesitation, while perhaps thoughtful, rests upon an insecure foundation, for it is doubtful whether any phenomenon associated with phagocytic research is any more rare than "superimposition." Those investigating the phagocytosis of highly virulent bacteria often have to search through as many as a hundred leucocytes before finding a single intracellular organism, although the smears are crowded everywhere with extracellular bacteria. It is a common experience in smearing exudates in infections involving highly virulent bacteria to find the smear a veritable carpet of microorganisms broken only occasionally by empty

leucocytes. The appearance of vacuoles surrounding the bacteria, slight modification in staining characteristics, distribution of organisms within the cell, and progressive disintegration of the bacteria associated with loss of staining qualities, are unmistakable criteria of ingestion, which reduce to a minimum the possibility of error in interpretation. The cases of phagocytosis reported in this paper were so energetic and thoroughly characteristic from every point of view that it is impossible to regard the phenomenon as any other than the engulfment and digestion of bacteria by active, living cells.

SUMMARY

Polymorphonuclear leucocytes sometimes take up and digest bacteria within the body after death. In one instance these phagocytes remained alive within the body for 11 days after death and were still capable of energetic phagocytosis.

Leucocytes suspended in physiological salt solution, provided a trace of serum be present, may remain alive and manifest active phagocytosis for a considerable time.

Marked changes in the temperature of the phagocytes after separation from the body do not destroy and often do not even alter the capacity of the leucocytes for phagocytosis.

Polymorphonuclear leucocytes which had retained their functional capacities for three days within the dead body were removed and after being kept several days in a test-tube were still capable of energetically phagocyting bacteria.

Phagocytes from the blood and exudates seem to possess about equal tenacity of function.

Human leucocytes are capable of sustained independent existence.

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THE INGESTION OF MELANIN PIGMENT GRANULES BY TISSUE CULTURES

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Introduction

In the course of a study of the origin and development of melanin pigment in cultures of the pigmented layer of embryonic chick retina, it was noted that fibroblasts growing in the same culture ingested the free pigment granules (Smith, 1920). This observation suggested the idea of adding pigment granules to cultures of tissues from other parts of the embryo, with the view of ascertaining whether the power to ingest these granules was limited to certain cells or was a property common to the cells of all the tissues. Accordingly, cultures of connective tissue, skin, lung, liver, kidney, endoderm of intestine, peripheral nerves, striated muscle, smooth muscle, and the pigmented layer of the retina were obtained. Melanin pigment granules were secured from the eyes of the chick, the pig, the dog, and a new-born child. In addition to these, an amorphous precipitate of melanin, isolated from the urine of a man suffering from a melano-sarcoma, was employed in the experiments.

I wish to express my thanks to Mrs. Lewis and Prof. W. H. Lewis, in whose laboratory this study was conducted; also to Prof. D. Wright Wilson, of the Department of Physiological Chemistry of the Johns Hopkins Medical School, for assistance in isolating the pigment compound from the urine.

Technique

Small pieces of the tissue to be studied were explanted in Locke-Lewis solution. When these cultures were 24 to 48 hours old, a drop of fluid containing pigment granules from the eye of a child, pig, dog, or chick was added. The granules were obtained by teasing the retinal layer in a drop of sterile Locke-Lewis solution, the pigment imparting a light brown color to the medium. A little of the amorphous pigment, previously sterilized, was likewise added to a few drops of sterile Locke-Lewis solution and a small drop of the mixture placed on a number of cultures. Other cultures were explanted directly into media containing these different types of pigment granules.

The cultures were incubated at 39°C. and studied in a warm box. Some were fixed at various stages in Zenker's solution without acetic acid and stained with iron hæmatoxylin.

Observations

Pigment Granules.—The melanin pigment granules from the chick's retina were in the form of short rods and between dark brown and black in color. Their specific gravity was probably slightly higher than that of distilled water, as they settled out quite readily. They remained suspended, however, in Locke-Lewis solution. In the hanging drop of the cultures they exhibited two distinct varieties of movement. One of these apparently corresponded to what is known as Brownian movement; the granules furthermore actually progressed from place to place with remarkable rapidity. At first glance this second type of motion resembled that shown by an actively motile colon bacillus; on closer observation, however, it could be seen that frequently the granules whirled end-over-end as they travelled. The changes in their course were somewhat angular as compared with the more sinuous movements exhibited by the colon bacillus.

The amorphous pigment from the urine likewise remained suspended in Locke-Lewis solution and exhibited Brownian movement, but did not move from place to place. The granules from the eye of the pig and of the child behaved in exactly the same manner as did those of the chick. They were about the same size and form, except that their ends were slightly more pointed or cigar-shaped. The granules from the eye of

FIGS. 1, 2, 3.—Tissue culture cells fixed in Zenker's solution without acetic acid. Stained with iron hæmatoxylin.

FIG. 4.—Cell from human liver fixed in Zenker's solution. Stained with hæmatoxylin and eosin.

All figures were drawn with camera lucida; No. 6 ocular and 2 mm. lens.

FIG. 1.—Normal pigmented cell from a 48-hour culture of the retina of a 5-day chick embryo. Each granule is an individual and discrete body of about the same size and shape. There are no vacuoles.

FIG. 2.—Fibroblast from a 48-hour growth of the subcutaneous tissue of a 7-day chick embryo. This cell has ingested melanin pigment granules from the retina of a 12-day chick embryo. Most of the granules are free in the cytoplasm; several are clumped together into pairs; and vacuoles are beginning to form about two of them.

FIG. 3.—Endothelial cell from a 96-hour growth of the liver of an 8-day chick embryo; the pigment within this cell is melanin pigment granules obtained from the retina of a pig. Two or three granules are free in the cytoplasm; other somewhat swollen granules are enclosed in vacuoles; several vacuoles are filled with fragmented granules; while others contain black homogeneous material. Near one side of the cell is a small irregular mass of finely granular debris.

FIG. 4.—A Kupffer cell from the sinusoids of the human liver. This cell has ingested malarial pigment. The masses of pigmented material are irregular in size and shape and a vacuole surrounds some of them. They resemble the ingested and partially destroyed pigment seen in Fig. 3.

the dog were found to be a little lighter in color than those of the chick and of four varieties: (1) small rods, similar in size and shape to those of the chick; (2) small round granules; (3) very large, oval or egg-shaped granules; (4) long spike-like granules over twice the length of those found in the chick's eye. The last three types were readily distinguishable from the normal pigment granules of the chick. The *dog* granules displayed essentially the same movements as the latter, but were probably a little heavier, as shown by their tendency to settle out of the drop.

The pigment appeared to be non-toxic, as no difference in the extent of growth of the tissue-culture was noted, even when the granules were so abundant as to color the medium.

In view of the fact that abundant fibroblasts were easily obtained from cultures of subcutaneous tissue, these cells were used to study the details of the ingestion process.

Fibroblasts.—Observations made immediately after the culture was treated with the Locke-Lewis solution containing pigment granules showed that a few of the granules had fallen on the tissue when the drop was applied, while the remainder were moving rapidly about in the fluid. As is usual in tissue-culture growths, the cells had migrated out on the under surface of the coverslip, so that during the procedure of adding a drop of pigment fluid some of the granules naturally settled on the cells, which, being rather sticky, held them. When the coverslip was reversed in returning it to the vaseline ring, these granules remained adherent to the under surface of the cell and were the first to enter it. Some of them were taken into the cytoplasm as early as 45 to 50 minutes after being introduced into the culture fluid. The free granules continued to move about in the fluid until they too became accidentally attached to the cells. When the granules were added directly to the culture medium at the time of explanting the tissues they remained suspended in the fluid until the cells grew out; then, as they moved about, some of them became attached to the surface of the cells and were subsequently taken into the cytoplasm.

In only a few instances did the cell make any active movement toward taking in the granule. This activity was shown by the slight bulging of the cytoplasm rather than the sending out of true pseudopodia. More often, however, the process would impress one as a gradual *sinking* of the granule into the cytoplasm. This, of course, is illusory, as the granule entered the cytoplasm from the under surface of the cell and could not *sink* upward. All the granules that adhered to a cell did not necessarily enter it. Some, for instance, after lying motionless under the cell for several minutes, or even a half hour, disengaged themselves and wandered off into the culture fluid, exhibiting the typical free-granule movement described above. Others, under apparently similar conditions, moved slowly up into the cytoplasm of the cell.

Although the pigment granules frequently entered the cytoplasm of cells containing vacuoles, they did not invade these bodies. Later vacuoles formed around the granules (Fig. 2), but the granules did not find their way into pre-existing vacuoles.

When free in the culture fluid the pigment granules exhibited the two kinds of movement described above: *i. e.*, Brownian and the progressive, end-over-end motion. After being taken into the cytoplasm of the cell, however, they showed the type of activity displayed by them when native in the pigmented cells of the retina (Fig. 1): *i. e.*, they started off with a spurt, proceeded a short distance, stopped suddenly and after a second or two of rest continued on. They sometimes moved back and forth in one region; again, they travelled entirely across the cell, passing either over or under the nucleus without apparently meeting any obstruction. When a granule first entered a cell it moved about freely, but usually within 3 to 24 hours a vacuole formed about it, due, perhaps, to the irritating effect of the granule on the cytoplasm. At first the vacuole was extremely small, appearing only as a slight halo around the granule (Fig. 2), the halo gradually enlarging into a definite vacuole (Figs. 2 and 3). For a time the granule continued its characteristic motion, carrying the vacuole about with it, but these movements became less and less extensive. As the vacuole increased in size, its contained fluid became much more refractive, so that the body was sharply marked off from the surrounding cytoplasm. When it became sufficiently large the granule within it exhibited Brownian motion. As the granule danced about in the vacuole, it began to swell (Fig. 3), tended to lose its rod shape, and appeared distinctly lighter in color. Whether this was due to an actual destruction of the coloring matter or merely a dilution of it, caused by the swelling of the stroma, could not be determined. Shortly after this the granule broke up into fragments. When this process of intracellular destruction had gone on for three or four days, there remained in the vacuole a small quantity of minute particles of debris.

Very often three or four granules appeared to have been agglutinated at one point in the cell, generally in the region of the centriole (Fig. 2) in which case one vacuole formed about them all. They all exhibited Brownian motion, gradually became swollen, lost their rod shape, and underwent the same process of disintegration and destruction as described above (Fig. 3).

This phenomenon could be studied to the best advantage with the aid of neutral red added to the cultures at the same time as the granules. When strong neutral red was used, the stroma of the melanin granules became stained (Smith, 1920) and remained red even when the granules were attached to the cytoplasm, although the cytoplasm in contact with them displayed no color. In most instances a weak solution of neutral red was used, and in these the free pigment granules remained unstained; even when they became attached to the cell, neither they nor the cytoplasm showed any evidence of staining. When vacuoles or neutral-red granules were already present in the cell, these at once took the stain, while the other components of the cell remained colorless. As stated above, when a pigment granule penetrated a cell it did not enter one of these pre-formed vacuoles, but moved back and forth in the colorless cytoplasm. Very soon, however (10 to 30 minutes), a narrow pink zone could be seen extending around the periphery of the granule. This was the intial stage in the

formation of the vacuole and could not be made out without the aid of the neutral red. The pink band grew gradually wider and deeper in color until there resulted a relatively large vacuole stained bright red. The stroma of the granule within the vacuole now became red also and retained this color throughout the entire process of dissolution. Even more striking was the fact that the debris resulting from the destruction of the granule retained the red color, even after fixation in Zenker's solution and staining with iron hæmatoxylin.

Although in some cases as many as twenty pigment granules were seen in a single fibroblast, it was not usual for these cells to contain more than about eight. The penetration by one granule semed to predispose the cell to further invasion; or it may have been that the cytoplasm of that particular cell was in a state to admit the granule more easily. For instance, one cell might have six or eight granules, while another cell growing beside it had none. There was nothing in the appearance of the cells in question to explain this difference in reaction. The entrance of granules into a cell evidently had some deleterious effect upon it, as such cells were observed to form degeneration vacuoles more frequently than cells in the same culture that had not taken in any granules.

No difference in behavior was noted between fibroblasts grown from the various tissues, whether subcutaneous, kidney, or lung tissue.

Clasmatocytes.—A certain number of clasmatocytes were nearly always present in these cultures, regardless of the kind of tissue from which the explant was made. These were large migrating cells whose cytoplasm contained numerous granules and fat globules. There were usually several large vacuoles present in these cells, even in healthy cultures in which no fibroblasts or other cells contained vacuoles. The clasmatocytes were characterized by numerous finger-like processes reaching out in all directions. They curved and twisted down into the hanging drop more often than they extended along the coverglass. While these pseudopodia displayed great activity, they never seemed to flow out like those of leucocytes or amœbæ.

The pigment granules appeared in clasmatocytes in large numbers and much more rapidly than in the fibroblasts. The phenomenon began almost immediately (10 to 20 minutes), and by the end of two hours many of these cells appeared quite black with granules, while the neighboring fibroblasts contained only a few. Individual clasmatocytes were observed to take in 30 or 40 granules. Some of the cells into which a large number of granules entered died shortly afterward. In preparations stained with neutral red this was indicated by the fading out of the stain from the vacuoles.

The granules did not move about from place to place in the clasmatocytes as freely as in the fibroblasts, but the vacuoles appeared to form with even greater rapidity. After a few hours a number of irregular masses of melanin were present in the cells. A vacuole was not readily distinguishable about some of these masses and in a few cases seemed to be entirely lacking. The process of decomposition of the melanin granules was essentially the same as in the fibroblasts, except that the destruction of the granules was not so complete.

Bloods-Cells.—Many of the cultures contained both red and white blood-cells. No pigment granules were observed within the red blood-cells, nor were any seen to be taken in by them. Generally, the white blood-cells were slightly more active than the fibroblasts but not nearly so active as the clasmatocytes. After the granules entered, vacuoles formed about them and the process of destruction proceeded as described above.

Epidermal Cells.—In view of the fact that in animals the epidermis is so often pigmented, cultures from that tissue of the chick were studied with considerable interest. About the same number of melanin granules were found in the epidermal cells as in the fibroblasts of the same culture. The vacuoles were more tardy in forming around the granules, but the granules were broken up eventually into finer particles than in the fibroblasts. In all other respects the processes were identical.

Alveolar Cells of the Lung.—Excellent growths from the cells forming the alveoli of the embryonic lung were obtained. The behavior of these cells toward the pigment granules was practically the same as that of the fibroblasts.

Liver Cells.—As a rule, the liver cells did not take in the granules in quite such large numbers or so rapidly as the fibroblasts. In these cultures certain other cells which resembled to some extent clasmatocytes but which were possibly primitive Kupffer cells, did, however, ingest the granules in large numbers and with considerable rapidity. In the liver cells, also, the granules were taken directly into the cytoplasm and later vacuoles formed about them. Bile was present in these cells but was not observed in the vacuoles that formed about the pigment granules. So far as could be made out, the granules were more nearly destroyed in the liver cells than in any others.

Endoderm of the Intestine.—In cultures of the intestine the fibroblasts grew out in strands, while the endoderm grew only in sheets. No essential difference was noted between the endodermal cells and the fibroblasts in their reaction toward the pigment granules. The granules were taken in, vacuoles formed about them, and they became broken up into irregular clumps of debris.

Kidney.—Cells of the kidney tubule grew out in the form of a thin membrane much like that observed in the liver cells. The pigment granules were taken into these cells and became broken up into fine particles similar to those found in the skin cells.

Striated Muscle.—In explants of striated muscle the cells grew out as long, spike-like strands containing many nuclei. No pigment granules were found within these muscle-fibers.

Smooth Muscle and Epithelium from the Amnion.—Two types of cells were obtained from explants of the amnion: epithelial cells which grew out in the form of a membrane, and smooth-muscle cells which grew out individually like the fibroblasts. The epithelial cells ingested granules in large numbers, being exceeded in this respect only by the clasmatocytes. Vacuoles did not form about the granules in these cells so rapidly or so extensively as in cells of other types. Some of the granules remained free in the cytoplasm for 24 hours or longer. The granules were eventually broken up into

fine particles, as noted in the ectodermal cells and in the cells of the kidney tubules. They were not found in clumps within these cells.

The smooth-muscle cells did not take in the granules when they first grew out nor as long as they were exhibiting rhythmical contraction; but when the cells had become spread out like fibroblasts, granules entered, vacuoles formed about them, and they disintegrated, just as in true fibroblasts.

Nerve Cells.—Sympathetic nerve cells were seen in cultures from various tissues. These were always free from pigment granules.

Pigment Cells.—Since pigment granules were taken in by many kinds of cells, it was deemed of interest to see if the pigment cells of the retina were themselves capable of ingesting foreign pigment granules. Granules from the dog's eye were added to a number of membranes of pigment-bearing cells from the retina of the chick, for the reason that these could be easily distinguished from the native pigment granules. They showed the same peculiar movement in the culture fluid as did the granules of the chick, but most of them sank to the bottom of the culture drop. In order that the pigment granules might be brought in contact with the growing cells, the cultures were incubated upside down for 12 hours. At the end of that time the majority of the cells had taken in large numbers of the foreign pigment granules. These were usually collected within one large vacuole and there was some evidence of their destruction.

Pigment Cells of the Fish.—While studying cultures from the embryo of the fish (Fundulus) Mrs. Lewis noticed that many of them contained pigment cells. A few of these cultures she kindly fixed for me. It could be readily seen that here we were dealing with two distinct types of cells containing pigment. The first was a rather large cell with numerous branched processes. This was the true chromatophore which is so common in reptiles, amphibians, and fish. These cells were snugly filled even to the ends of their processes with more or less rounded granules, each of which could be made out as a separate unit. In some of these cells the granules were black, in others yellow, reddish, or light brown; but the striking fact was that no single cells had granules of more than one color. The second type of cell containing pigment granules was the mesodermal cell, which frequently contained granules of more than one color. These were clumped together in vacuoles, exactly as in the cells of the chick embryo described herein. This evidence all points to the fact that these cells had ingested and not formed the granules.

DISCUSSION

From my studies on the behavior of the cells of tissue cultures toward the pigment granules, when the two structures are brought together under experimental conditions, it seems to me that the melanin granules have special advantages over other materials used in the study of ingestion. Their dark color makes them easy to follow, and one can tell from their behavior exactly what part of the process is going on; *i. e.*, when the granule is free in the culture fluid, it exhibits both Brownian motion and actual progression; when attached to the cell, it is motionless; when free in the cytoplasm, it displays the jerky motion characteristic of the native pigment granules; and finally, when surrounded by a vacuole, it again undergoes Brownian motion.

The method by which the melanin granules make their entrance into a cell invites speculation as to the factors involved in the phenomenon. Certainly it appears to be very different from the manner in which an amœba is supposed to engulf its food. The cells do not send out pseudopodia and surround the granules;[1] nevertheless, from all appearances, it seems quite certain that the process depends more upon the cytoplasm than upon the activity of the granules themselves. From my observations there would seem to be at least two stages in this process. In the first the granule becomes attached to the side or under surface of the cell; in the second stage it enters the cell and moves about freely within the cytoplasm.

The question arises: How does the granule get from the external surface to the interior of the cell? It seems most probable that the phenomenon of capillary attraction is the basic principle, but in the application of this principle we have two possibilities to consider: In the first place, is the process simply a pulling in of the granule once it is in intimate contact with the cytoplasm? Or, must there be some local modification of the cellular wall before the factor of capillary attraction can come into play? The varying lengths of time required for the completion of the process in different types of cells point to the latter as the more probable solution.

A large variety of cells have been shown to possess the power of ingesting foreign material. A long series of dyes (Evans and Schulemann, 1914, Clark and Clark, 1918), carbon granules (MacCallum, 1903), fine particles of certain metals (Plato, 1900), melanin pigment granules (Matsumoto, 1918), and also bacteria (Metchnikoff, 1905, Plato, 1900; Evans, 1915; Jones and Rous, 1917; Lewis, 1920) have been used in the study of this phenomenon. While certain of these observers occasionally mentioned vacuoles in connection with the ingested foreign body, it had not been definitely stated that vacuole formation always accompanied such ingestion until 1919, when Shipley stated (page 293) that "all sorts of material which the cell allows to enter, or takes into, its cytoplasm, eventually finds its way into some of these vacuoles of segregation." On page 297 he asserted: "When large foreign bodies are ingested, they are enclosed in a fresh vacuole formed during their engulfing."

However, in the case of the melanin granule and also the avian tubercle bacillus,[2] it was clearly evident that the foreign

[1] Dr. Mary Hogue, of the School of Hygiene, Johns Hopkins Medical School, noted that amœbæ in tissue cultures ingested melanin pigment granules obtained from the eye of an embryo chick. This particular species of amœba did not "ingulf" the pigment granules, neither were the granules taken into food vacuoles, nor were vacuoles formed about them coincident with their entrance into the cell.

[2] Certain experiments were undertaken in order to determine whether cells ingest and destroy bacteria in the same manner in which they take in and destroy pigment granules. Suspensions of avian tubercle bacilli, obtained through the kindness of Dr. H. S.

body was not taken into a preformed vacuole, nor did a vacuole form about it coincident with its entrance into the cell, as claimed by Shipley; it was only after the granule had been in the cytoplasm for some time that a vacuole was formed about it by the cytoplasm. This vacuole may have contained digestive enzymes, oxidizing agents, or other substances which acted upon the melanin in such a way as to cause it to break up into particles. It was evident from the neutral red reaction that the vacuole was slightly alkaline. The protein composing the stroma of the melanin granules is one which does not easily undergo decomposition, as has been shown by Abel and Davis (1896), who found that these granules were so resistant outside the body that they were not destroyed by concentrated HCl, and were broken up only after prolonged treatment in the water-bath in dilute (5-10 per cent) KOH.

In this and previous studies it was observed that the granules within any particular true pigment-producing cell were always individual and discrete bodies of about the same size, color and shape (Fig. 1). Individuality and discreteness were common properties of the granules in all true pigment-producing cells, but color, size and shape varied according to the origin of the cell. The granules were never found clumped together or in vacuoles in these cells. On the other hand, granules or other particles of pigmented material, when ingested, were usually found clumped into masses of irregular size and shape, enclosed in vacuoles, or broken up into debris (Figs. 3 and 4).

Combining these two observations, it can be seen that here is a possible method of determining whether the pigment granules within a certain cell were produced by that cell or were merely taken in by it from the surrounding medium. Several hundred sections of tissue, both normal and pathological, including a large number of pigmented tumors, have been examined, and the indications are that this criterion will enable us to settle the old question of pigment-producing (Fig. 1) versus pigment-carrying cells (Figs. 2, 3, and 4). In a given pathological condition (Fig. 4), where pigment is present, one can tell whether the cells are producing or ingesting it. In the case of tumors one can distinguish between the true pigmented tumors (those arising from pigment-bearing cells) and those in which pigment occurs as a result of the cells ingesting melanin pigment, blood pigment, or both.

Summary

(1) Melanin pigment granules from the retina of the chick, the pig, the dog, and man (child) showed a variety of movements which enabled one to identify the position of the granule, *i. e.*, to determine whether it was free in the culture fluid, adhering to the cell, within the cytoplasm, or within a vacuole.

(2) Flakes of an amorphous precipitate of impure melanin pigment, isolated from the urine of a man suffering with extensive metastases from a melano-sarcoma, showed Brownian motion but did not progress from place to place.

(3) Fibroblasts, clasmatocytes, endothelial cells, white-blood cells, epidermal cells, alveolar cells of the lung, liver cells, kidney tubule cells, endodermal cells of the intestine, pigment cells of the retina, flattened out smooth-muscle cells, and epithelial cells of the amnion of the chick ingested melanin pigment granules.

(4) Red-blood cells, striated-muscle cells, rhythmically contracting smooth-muscle cells, and certain peripheral nerve cells, occasionally found in the cultures, did not ingest the granules.

(5) The pigment granules were taken directly into the cytoplasm of the cell and not into preformed vacuoles; as the granule moved back and forth in the cell, however, a vacuole formed about it. Within the vacuole the granule exhibited Brownian motion. Gradually, it was reduced to tiny particles of debris. Frequently a number of granules became clumped together, one vacuole forming about them all. The clumping of the granules together into large masses did not take place so extensively in the ectodermal or epithelial cells, but in these each granule was broken up into very fine particles.

(6) Mesodermal cells of the fish embryo (Fundulus) ingested pigment granules liberated from the chromatophores of the fish.

(7) Native granules in the true pigment-producing cells are always individual and discrete bodies of about the same size and shape. They are never found clumped together in vacuoles or broken up into fragments. It can thus be determined by the appearance of the granules whether they have been produced within the cell in which they are seen or have been ingested.

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Willis, of the Kenneth Dows Tuberculosis Research Laboratory, Johns Hopkins Hospital, were placed upon tissue cultures. It was soon evident that the manner in which the bacilli entered the cell and the vacuoles formed about them was much the same as that exhibited by the melanin granules. The details of these observations will be described in a separate publication.